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14. ABSTRACT
In the second year, a cutaneous wound model in rats with the drug resistant Gram negative bacteria *Acinetobacter baumannii*, *Escherichia coli*, *Klebsiella pneumoniae* and pathogenic mold *Aspergillus fumigatus* was established. Efficacy of CHD-FA against cutaneous wounds infected with these organisms was assessed respectively. Significant wound surface area reduction with high and mid CHD-FA treatment doses was observed with all studied infection models. Remarkable bioburden reduction induced by CHD-FA was also observed in wounds infected with multidrug resistant *E. coli* and *K. pneumoniae*. To better assess wound healing upon CHD-FA treatment at cellular and molecular level, a more comprehensive histopathological evaluation and expression profiling of wound healing genes was performed at various time points during the infection/treatment course in rats infected with MRSA and *P. aeruginosa*, respectively. Histopathological analysis showed that the infection and inflammation in the wounds from both infection models treated with CHD-FA was better controlled relative to the untreated and antibiotic control groups, as indicated by lower neutrophils scores as early as day 3. The decrease in cellular inflammation and increase in epithelialization at the endpoint of the experiment suggested wound healing was significantly improved with CHD-FA treatment. Host gene expression profiling displayed the same dynamic trend of differentially expressed wound healing genes upon CHD-FA treatment in both infection models. The levels of overexpression were found to be more prominent in the CHD-FA group at day 3 compared to the untreated control, and their expression levels rapidly returned towards baseline at day 6, as the same set of genes in the untreated control increased. In particular, the key biomarker of impaired wound healing IL6 was constantly overexpressed day 3 to day 6 in the untreated wound. In contrast, expression of IL6 was significantly dampened at day 6 in the CHD-FA treated wounds, indicating an accelerated and better controlled wound healing. A full preliminary evaluation of CHD-FA to treat wound infections with all the pathogens listed in the Statement of Work (SOW) has now been completed with the submission of this annual report. These preliminary results are encouraging and histopathological and host gene expression profiling on wounds infected will be performed with the other pathogens listed in the statement of work. Other wound infection models such as the burn model and deep tissue (thigh) model will also be performed in the final year.

15. SUBJECT TERMS
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Introduction

The objective of this study is to demonstrate the potent antimicrobial properties of carbohydrate-derived fulvic acid (CHD-FA) against a broad collection of drug-sensitive and multi-drug-resistant (MDR) pathogens commonly associated with wound infections, and assess the relative efficacy of CHD-FA against induced wound infections in *in vivo* animal models. Overall, this work is intended to establish CHD-FA as a safe and effective agent that can be deployed to prevent the onset of drug-resistant bacterial and fungal infections in military and civilian personnel suffering traumatic wound infections. Given its novel mechanism of action and preliminary activity against MDR bacteria and antifungal-resistant fungi, the early use of CHD-FA is advantageous because it represents a novel target that will not select for resistant organisms, and prevents the use of more specific but more limited spectrum antibiotics. The overall goal of this preclinical program is to establish a firm justification for progressing to human trials to determine the efficacy of topical CHD-FA in preventing wound infections in injured military personnel.

Body

Description of Overall Progress in the first Annual report. (Sept 2012 to Sept 2013).

Section 2. Description of Overall Progress

As described in the Annual Technical Report submitted on 10/29/2013, Specific Aim 1 of the statement of work establishing the *in vitro* efficacy of CHD-FA against drug resistant bacteria and fungi was completed. The minimum inhibitory concentrations (MIC_{50} and MIC_{90}) for CDH-FA against a collection of over 600 multidrug resistant Gram negative, Gram positive bacterial and fungal clinical isolates was determined. The results demonstrated the potent anti-microbial activity of CHD-FA against a broad range of drug resistant bacteria and fungi. Most bacteria show complete growth inhibition at 0.06 – 0.125% fulvic acid, while efficacy with fungi is 0.125-0.5%.

In the 3rd quarter report (Q3.07.2013), we established the cutaneous wound model in rats. This model was performed without infection in order to observe natural wound healing process and to establish the kinetics of such process in rats. In the same report we established a wound infection in rats with Methicillin Resistant *Staphylococcus aureus* (MRSA) strain Xen31 (specific aim 2) in a 10 day infection model. The wounds were prepared in the same manner as described in the section on establishing the wound model in Quarterly report Q3.07.2013. Wounds were infected with a final bacterial load of 5 $x10^8$, $1x10^7$, or $5x10^6$ CFU. To assess healing, daily digital wound measurement were taken with a Nikon 4x Stereomicroscope with FS-1 digital camera. Wound closure as percentage of wound surface area was discernible between the highest infection dose and the lower doses from days 6 through 8, however by the experimental endpoint at day 10 the untreated infected wounds were all similar in surface area. Purulent discharge was observed in the wounds, but there were no observable signs of sepsis in any of the infection doses evaluated. The final infection dose for MRSA was $1x10^8$ CFU for drug treatment studies was decided based on the untreated wound healing kinetics. A wound infection in rats with multi-drug resistant Gram negative bacterium *Pseudomonas aeruginosa* strain Xen05 was also established in the Annual Technical Report following the same methodology with MRSA. In this infection model, evidence of hemolysis in the wounds was observed by day 1 as well as purulent discharge throughout the study. The highest infection dose of *P. aeruginosa* resulted in rats becoming moribund by day 2 and they were humanely euthanized. Bacterial dissemination to the peripheral organs was observed during post mortem analysis of the rats. A final infection dose of $1x10^7$ CFU was used for all drug treatment studies as it provided a more consistent wound healing kinetics.

In the 3rd Quarter and Annual Technical Reports we evaluated CHD-FA to treat cutaneous wounds infected with MRSA and *P. aeruginosa*. Daily wound closure measurements and bacterial burdens in wounds at the experimental endpoint (10 days) were the metric used to assess treatment efficacy. For both treatment studies 4.6%, 0.46% and 0.046% of CHD-FA was applied to the wound sites daily at 4 hours. For MRSA, a 24 hour post infection treatment start time was also used to assess if a delay in treatment would significantly affect wound healing kinetics. The MRSA infection was a more appropriate model to evaluate this as the virulence study demonstrated the rats could better tolerate MRSA wound infections. In the treatment efficacy studies for MRSA, improved wound closure was visually observed in the highest and middle CHD-FA doses (4.6 and 0.46%) relative to the untreated control in both the 4h and 24h post infection treatment start times. Purulent discharge was observed in some of the CHD-FA treated and untreated rats regardless of the treatment start time (24h or 4h, post infection), however, the discharge was less pronounced in the CHD-FA treated groups. Digital measurements of wound surface areas showed significant improvement in wound healing in the 4h treatment start time than the 24h start in all 3 doses evaluated. The most prominent differences were on day 7 where the average wound surface areas for the 24h treatment start were 56.8%, 59.1% and 64.4% in contrast to the 4 h treatment start of 43.9%, 45.3% and 43% for CHD-FA

doses 4.6%, 0.46% and 0.046%, respectively. The wound surface area percentages for the untreated controls on day 7 was 70.4% and 64.5%, for the 24h and 4h treatment start time, strongly suggesting the initiation of CHD-FA therapy is an important factor in improved wound healing. The bacterial burden assessment of the wounds at the experimental endpoint at 4h CHD-FA treatment time had 1.5, 2.1 and 0.3 log reduction in burden for the CHD-FA treatment doses 4.6%, 0.46% and 0.046% relative to the untreated controls.

For the *P. aeruginosa* treatment studies, improved wound closure was visually observed in the highest and middle CHD-FA doses (4.6 and 0.46%) relative to the untreated control. The greatest improvement in wound healing became evident on day 7. As expected, both the CHD-FA treated and untreated wounds were significantly healed by the experimental endpoint at day 10. Purulent discharge was observed in some of the CHD-FA treated and all of the untreated rats however, as similar to the MRSA treatment studies the discharge was less pronounced in the CHD-FA treated groups. Digital measurements of wound surface areas showed significant improvement in wound healing on day 7 with the highest (4.6%) and mid (0.46%) CHD-FA treatment doses. The most prominent differences were on day 7 where the average wound surface areas were 49.3%, 46.9% in contrast to the untreated control of 60.8%. The lowest CHD-FA (0.046%) treatment dose had wound surface area percentage of 61.2% on day 7, not significantly different from the untreated controls. However, less purulent discharge was observed for the low CHD-FA dose than the untreated controls. The bacterial burden assessment of the wounds at the experimental endpoint 1.0, 0.5 and 0.3 log reduction in burden for the CHD-FA treatment doses 4.6%, 0.46% and 0.046% relative to the untreated controls. As with the MRSA infection studies these promising results warrants further studies with other bacterial and fungal pathogens.

Description of Overall Progress during the current Annual report. (Sept 2013 to Sept 2014).

During the last 3 quarters, we continued working on specific aim 2 of the statement of work and established cutaneous wound model in rats with the drug resistant Gram negative bacteria *Acinetobacter baumannii*, *Escherichia coli*, *Klebsiella pneumoniae* and pathogenic mold *Aspergillus fumigatus*. We assessed the treatment efficacy of Carbohydrate-Derived Fulvic Acid (CHD-FA) against cutaneous wounds infected with these organisms respectively.

Section 3. Establishing the wound infection in rats with *Acinetobacter baumannii* strain ATCC BAA-747 (specific aim 2). Q5 Progress: 09/30/2013 through 12/30/2013

Our objective for the first quarter in the second year of this grant award is to continue to establish the cutaneous wound infection models and assess CHD-FA treatment efficacy with agents listed in Specific Aims 2 of the statement of work. In this quarterly report, we established the cutaneous wound model in rats with the drug resistant Gram negative bacterium *Acinetobacter baumannii* and pathogenic mold *Aspergillus fumigatus.* Preliminary CHD-FA treatment efficacy data that included wound closure measurements and enumeration of microbial burden on wound sites were also collected on these pathogens.

As with the previously reported virulence assessment studies (Q3 and Annual Technical Reports), 9 rats were randomized into three infection groups (3 rats per group). *A. baumannii* strain ATCC BAA-747 was inoculated in Brain Heart Infusion (BHI) media and incubated at 37ºC with shaking overnight. Bacterial cells were washed, precipitated in sterile PBS, and diluted to $2x10^9$, $2x10^8$, and $2x10^7$ colony forming units (CFU) per ml for the infection. Two wounds were created on each rat, for a total of eighteen wounds. The process of anesthetizing the rats and creating the wounds was the same as described above in the section on establishing the wound model and in quarterly technical report Q3.07.2013. After wound creation, rats from each challenge group were infected with 0.05 ml of the

cell suspension with corresponding doses. The final infection doses for the rats were $1x10^8$, $1x10^7$, and $1x10^6$ CFU, respectively.

All the wounds infected with *A. baumannii* became purulent on days 1 or 2. The discharge of the wounds was observed at various infection doses throughout the 10-day experiment but was more pronounced in the higher infection doses. Initiation of scab formation was observed by day 1 and all wounds had scabs by day 2 **(Fig.1)**. Wound closure in the rats was noticeably visible starting at day 6 at the two lower infection doses ($1x10^7$ and $1x10^6$ CFU) and by day 7 at the highest dose ($1 x10^8$). There were no observable signs of sepsis such as lethargy or recumbency as a result of infection in any of the rats. Daily digital wound measurement were taken with a Nikon 4x Stereomicroscope with FS-1 digital camera. Wound closure as percentage of wound surface area **(see Fig.2)** was discernible between the highest infection dose and the lower doses from days 6 through 8, however by the experimental endpoint at day 10 the untreated infected wounds were all similar in surface area measurements. The wound closure measurements over a 3 log range of infection doses simulating geometric progression suggest this pathogen causes a more persistent wound infection that is not solely dependent on bacterial population.

Conclusion

Based on the virulence study, a $1x10^7$ CFU infection dose will be used for all downstream *A. baumannii* cutaneous wound infections

Fig. 1. Cutaneous wounds in rats with *A. baumannii* (ATCC BAA-747) infection.

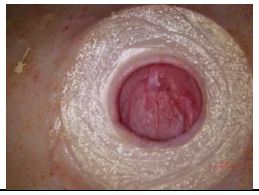
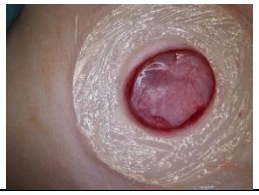
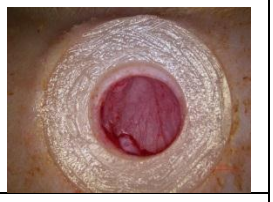
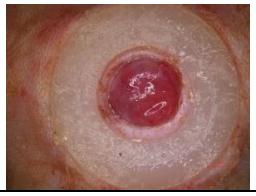
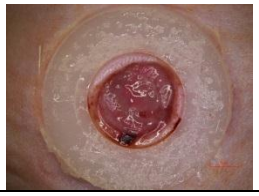
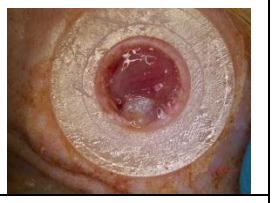
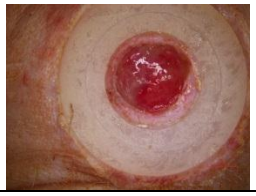
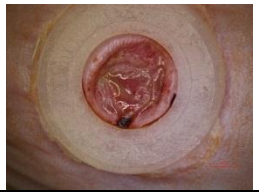
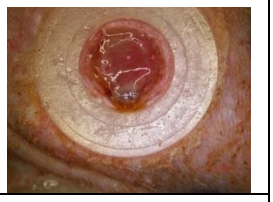
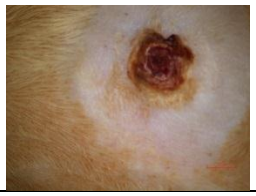
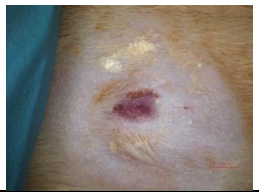
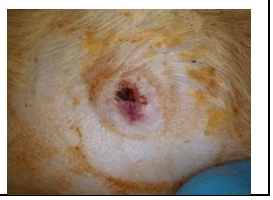
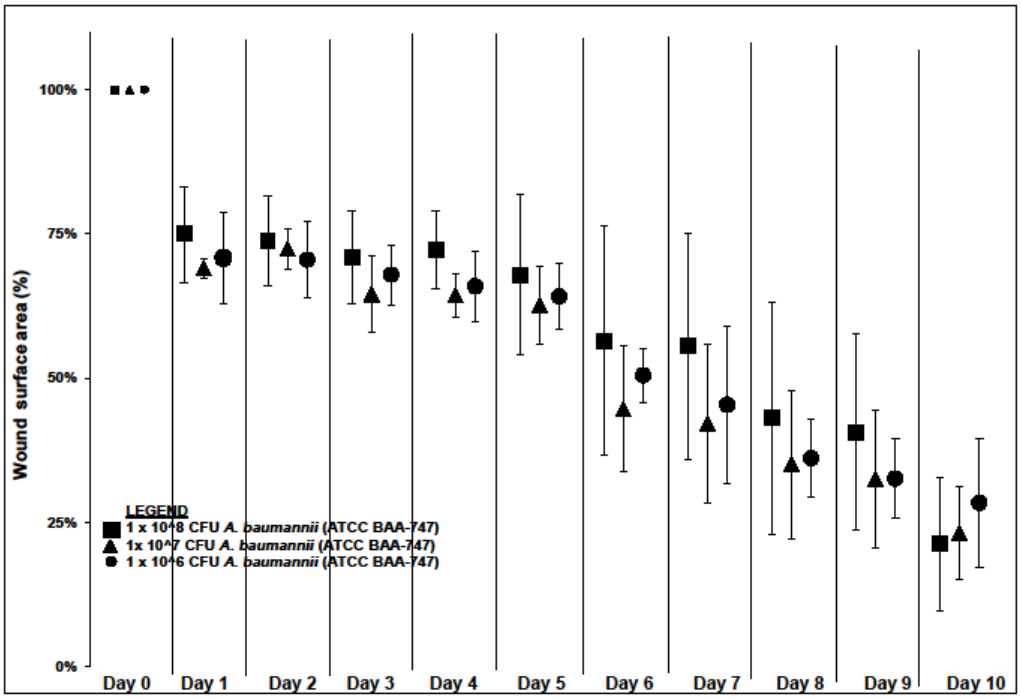
Day	Rat #2 ($1x10^8$ CFU)	Rat #5 ($1x10^7$ CFU)	Rat #7 ($1x10^6$ CFU)
0			
1			
2			
10			

Figure 2. Wound surface area plot of wound infection model with *A. baumannii*



Section 4. Establishing the wound infection in rats with *Aspergillus fumigatus* strain R21 (specific aim 2).

A total of 12 rats were randomized into four infection groups (3 rats per group). *Aspergillus fumigatus* strain R21 was grown in semi-solid Potato Dextrose Agar media and incubated at 37ºC for 4 days to allow for sporulation. Spores were isolated from the agar plate by gentle scraping with a sterile disposable scraper and irrigated with sterile saline containing 0.05% Tween 20. The crude spore suspension was transferred to a new sterile tube and left undisturbed for 10 minutes to allow the mycelia debris to settle to the bottom of the tube. The supernatant containing the spores was carefully collected and filtered through a sterile Miracloth. The spore suspension was washed twice in sterile PBS, and diluted to $2x10^{10}$, $2x10^{9}$, $2x10^{8}$ and $2x10^{7}$ spores per ml for the infection. Two wounds were created on each rat, for a total of eighteen wounds. After the wound creation, rats from each challenge group were infected with 0.05 ml of the spore suspension with corresponding doses. The final infection doses for the rats were 1 $x10^{9}$, $1x10^{8}$, $1x10^{7}$ and $1x10^{6}$ spore, respectively. A broad four log dilution of *A. fumigatus* spores for the virulence assessment in rats was chosen as there was no existing data on cutaneous infections in rodents with *A. fumigatus*.

All the wounds infected with *A. fumigatus* became severely purulent on starting on days 1 or 2. The continual discharge of pus from the wounds was observed throughout the 10 day experiment. For the highest infection dose ($1x10^{9}$ spores), the wound site was blackened due to the high concentration of *Aspergillus* spores **(Fig.3)** and the dark hue remained even in the scab. Scab formation was observed starting on day 1 and all wounds had scabs by day 2. Wound closure in the rats became noticeably visible starting at day 5 at the two lower infection doses ($1x10^{6}$ and $1x10^{7}$ spores) and by day 6-7 at the second highest dose (1 $x10^{8}$). It was difficult to observe wound closure in the highest dose due to the dark pigmentation of the scabs. There were no observable signs of sepsis such as lethargy or recumbency as a result of infection in any of the rats. However, one of the rats given the highest infection dose was observed with a productive discharge from its mouth by day 2. Two rats given the highest infection dose developed a subcutaneous abscess between the wound sites on the flank. The

abscesses were aseptically excised at the end of the study and verified to contain *A. fumigatus.* A small mass central to the wound site was seen starting on day 6 in rats given 10^7 or 10^8 spores. The frequency and size of the mass was higher in rats given 10^8 spores. Upon gross examination, the mass appeared yellowish at 10^7 spores of infection but was more dark and greener at the 10^8 spore infection dose. Daily digital wound measurement were taken with a Nikon 4x Stereomicroscope with FS-1 digital camera. Wound closure as percentage of wound surface area **(see Fig.4)** was discernible between the 10^8 infection dose and the lower doses from days 6 through 8, however by the experimental endpoint at day 10 the untreated infected wounds were all similar in surface area.

Conclusion

Based on the virulence study, a $1x10^8$ spore infection dose will be used for all downstream *A. fumigatus* cutaneous wound infections.

Fig. 3. Cutaneous wounds in rats with *Aspergillus fumigatus* (R21) infection

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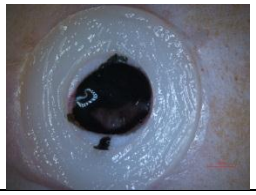
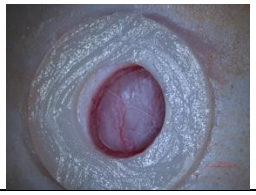
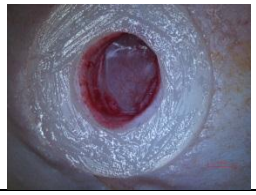
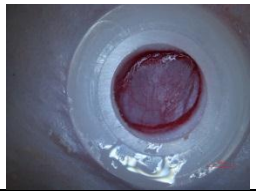
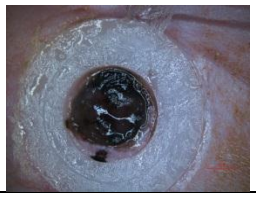
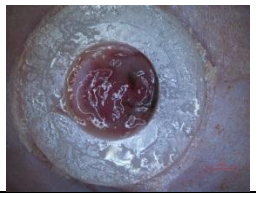
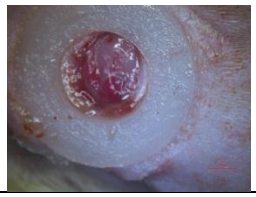
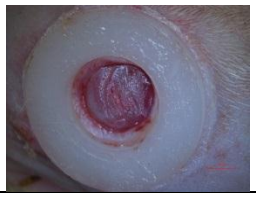
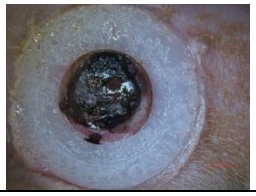
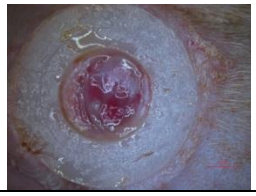
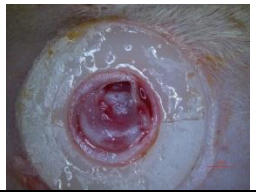
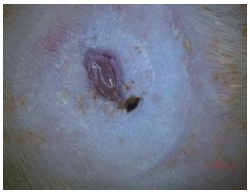
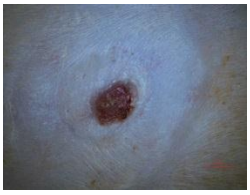
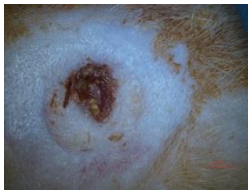
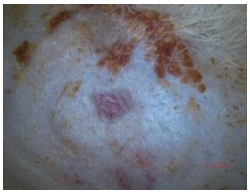
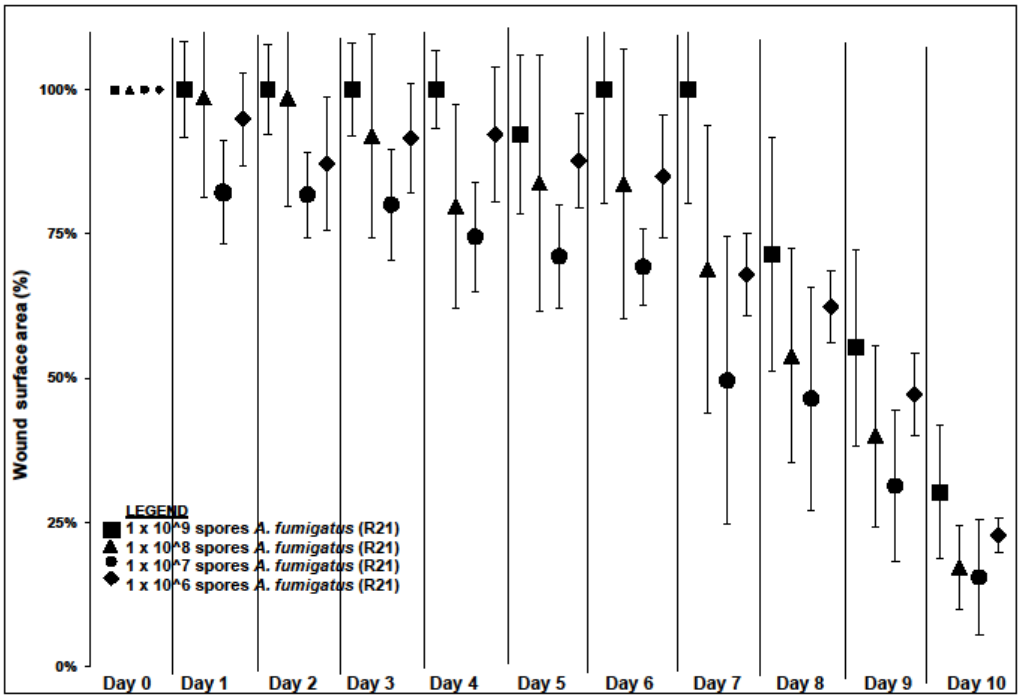
0				
1				
2				
10				

Figure 4. Wound surface area plot of wound infection model with *A. fumigatus*



Section 5 Evaluation of CHD-FA to treat cutaneous wounds infected with *Acinetobacter baumannii* strain BK (specific aim 2).

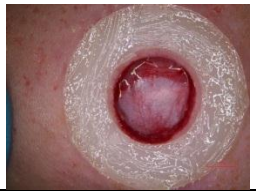
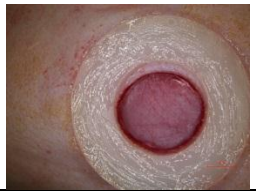
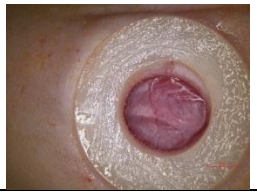
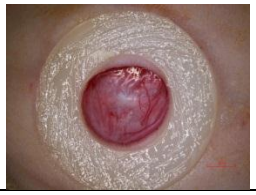
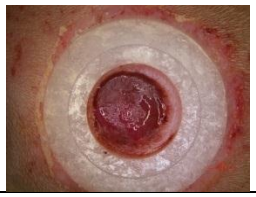
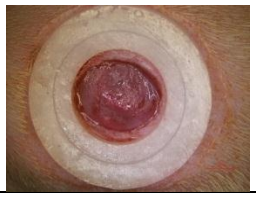
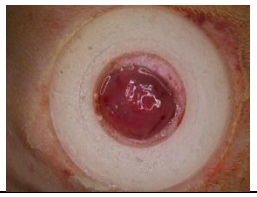
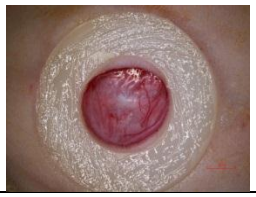
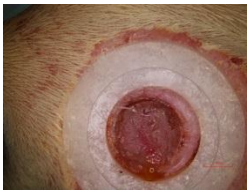
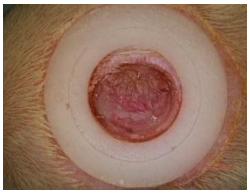
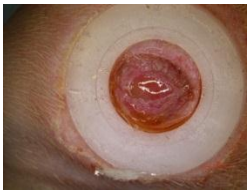
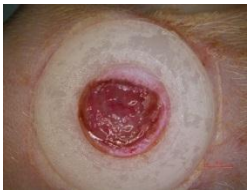
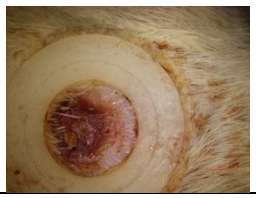
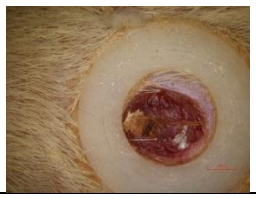
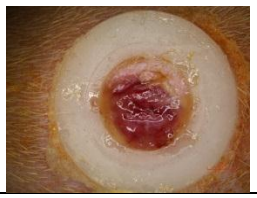
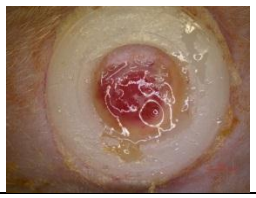
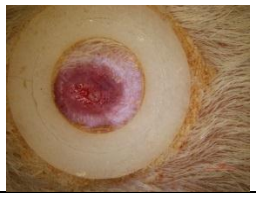
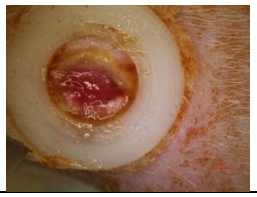
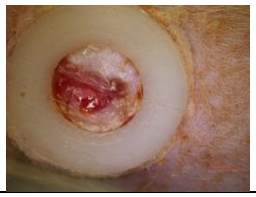
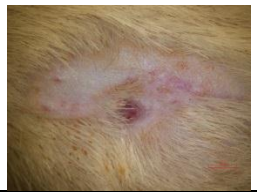
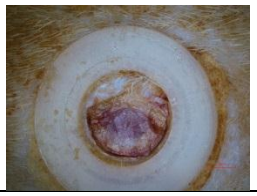
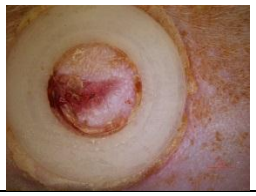
To assess the treatment efficacy of CHD-FA against cutaneous wounds infected with *A. baumannii*, 11 rats were randomized into three treatment groups (3 rats per group) and two untreated (sham) controls. A*. baumannii* ATCC BAA-747 was prepared at described in 2.a. Wounds were created and infected with 0.05 ml of *A. baumannii* to a final infection dose of $1x10^7$ CFU*.* Rats were given daily treatments of CHD-FA at 4.6, 0.46 or 0.046% in 0.025 ml volumes starting at 4 hours post infection.

Improved wound closure was visually observed in the highest and middle CHD-FA doses (4.6 and 0.46%) relative to the untreated control starting on day 7 **(Fig 5)**. The highest difference in wound closing was on days 8 and 9 of the infection. In our previous findings with other bacterial pathogens, the most improved wound closures were observed on days 5 to 7. The delay in treatment efficacy suggests a bacteriostatic activity of the compound, an unusual finding considering the dearth of in vitro data suggesting otherwise. As expected, both the CHD-FA treated and untreated wounds were significantly closed by the experimental endpoint at day 10. Purulent discharge was still observed in all of the rats, however the duration and amount of discharge in the CHD-FA treated rats at 4.6% and .46% were less than the untreated controls by1 to 3 days. Digital measurements of wound surface areas **(Table 1)** showed significant improvement in wound healing on days 7 to 9 with the highest (4.6%) and mid (0.46%) CHD-FA treatment doses. The most prominent differences were on day 9 where the average wound surface areas were 21.1% in the high dose group (4.6%) in contrast to the untreated control at 38.9%. The lowest CHD-FA (0.046%) treatment dose wound closure values were not significantly different from the untreated controls. However, less purulent discharge was observed for the low CHD-FA dose than the untreated controls. Interestingly, the bacterial burdens of the all wounds at the experimental endpoint were less than 2 logs. This could be attributed to the discharge. We will collect earlier time points of the wound burdens to address this.

Conclusion

As with the MRSA and *P. aeruginosa* infection studies there was significant reduction of the size and bacterial burden in the wound sites with high and mid CHD-FA treatment groups. These preliminary results are encouraging and we will be repeating these studies with a focus on the earlier time points to assess bacterial burden. Furthermore, we will also perform both histopathologic analyses and host gene expression profiling to better assess the cellular and molecular mechanisms during wound healing with CHD-FA.

Figure 5. Wound images of rats infected with *A. baumannii* (ATCC BAA-747) and treated with CHD-FA at 4h post infection.

Day	Rat #1 4.6% CHD-FA	Rat #6 0.46% CHD-FA	Rat #7 0.046% CHD-FA	Rat#11 Untreated
0				
1				
2				
5				
7				
10				

Section 6. Evaluation of CHD-FA to treat cutaneous wounds infected with *Aspergillus fumigatus* strain R21 (specific aim 2).

To assess the treatment efficacy of CHD-FA against cutaneous wounds infected with *A. fumigatus*, 11 rats were randomized into three treatment groups (3 rats per group) and two untreated (sham) controls. *A. fumigatus* R21 spores was prepared as previously described in 2.c. Wounds were created and infected with 0.05 ml of *A. fumigatus* R21 spores to a final infection dose of $1x10^8$ spores. Rats were given daily treatments of CHD-FA at 4.6, 0.46 or 0.046% in 0.025 ml volumes starting at 4 h post infection.

Improved wound closure was visually observed in the highest and middle CHD-FA doses (4.6 and 0.46%) relative to the untreated control. Closure of the wound was initiated on day 5-6 for the highest dose of CHD-FA (4.6%) and on day 6 for the middle dose (0.46%) **(Fig 6)**. Purulent discharge was evident in all rats throughout the study, however the duration and amount of discharge were less in the CHD-FA treated rats at 4.6% and 0.46% relative to the untreated controls. The wounds all displayed a small mass central mass with grey to greenish discoloration, indicating mold infiltration of the wound. Rats given the highest and middle doses of CHD-FA displayed smaller or no mass and very light grey discoloration of the wound and discharge in comparison to the lowest treated dose (0.046%) and untreated control groups. A more greenish color was seen in the untreated and lowest dose groups. One of the untreated controls died during anesthesia and prior to infection, leaving only one rat and therefore only a median value for wound closing could be provided. However, it should be noted that the wound closing kinetics for the untreated rats were very consistent to untreated rats in the virulence study. The digital wound measurements showed the greatest difference in wound closing was on days 5 through 8 of the study with the highest and middle CHD-FA **(Table 1)**. On day 8, the CHD-FA highest dose (4.6%) has a wound surface area of 32.6% and the untreated control was 54.5%. The lowest CHD-FA (0.046%) treatment dose wound closure values were not significantly different from the untreated controls. The fungal burdens of all the wounds at the experimental endpoint were less than 2 logs and thus no log reduction in burden could be determined. The poor recovery of *Aspergillus* in wound site could be attributed to the purulent discharge. We will collect earlier time points of the wound burdens to address this.

Conclusion

As seen with the previous bacterial infection studies there was significant reduction of the size in the wound sites with high and mid CHD-FA treatment groups. These preliminary results are encouraging and we will be repeating these studies with a focus on the earlier time points to assess fungal burden. Furthermore, we will also perform both histopathologic analyses and host gene expression profiling to better assess the cellular and molecular mechanisms during wound healing with CHD-FA.

Figure 6. Wound images of rats infected with *A. fumigatus* (R21) and treated with CHD-FA at 4h post infection.

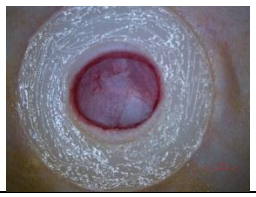
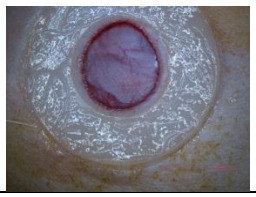
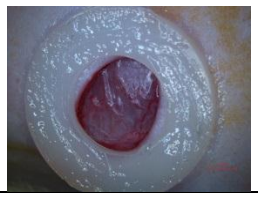
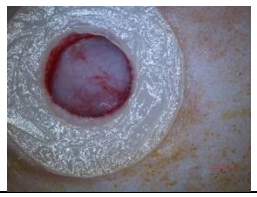
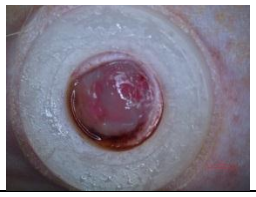
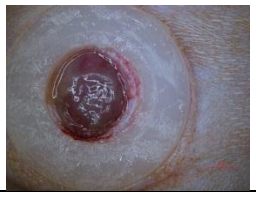
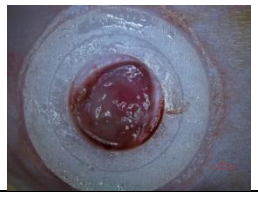
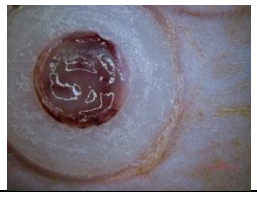
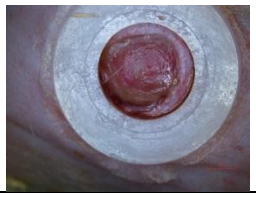
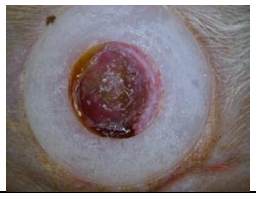
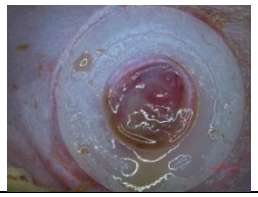
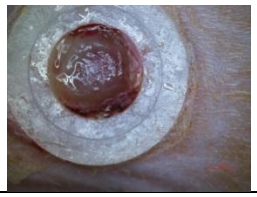
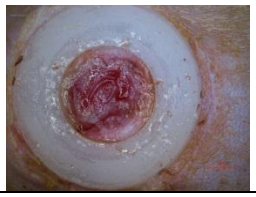
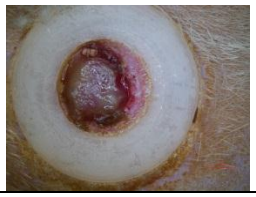
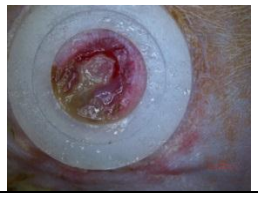
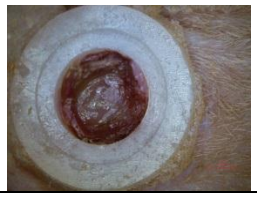
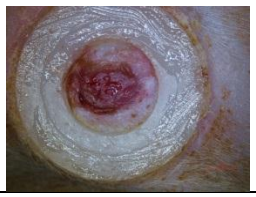
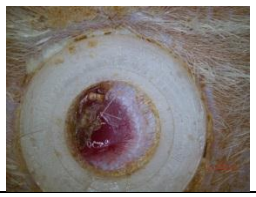
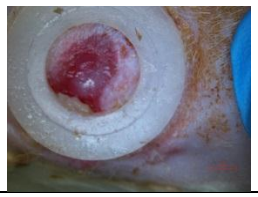
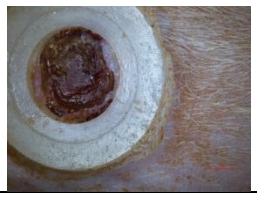
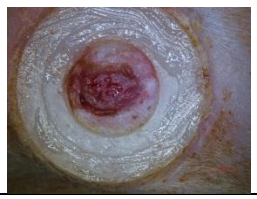
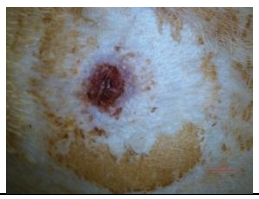
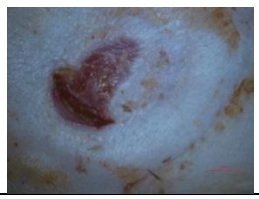
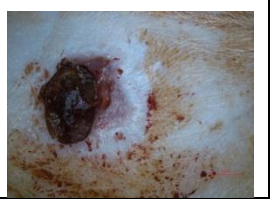
Day	Rat #1 4.6% CHD-FA	Rat #5 0.46% CHD-FA	Rat #9 0.046% CHD-FA	Rat#10 Untreated
0				
1				
2				
5				
7				
10				

Table 1. Wound closure analysis of Rats infected with *Acinetobacter baumannii* and *Aspergillus fumigatus* treated with CHD-FA.

A. bau	CHD-FA Treatment Concentration (%)							
4h Tx	4.6		0.46		0.046		No Tx	
Day	Wound surface area	SD	Wound surface area	SD	Wound surface area	SD	Wound surface area	SD
0	100.0%	n/a	100.0%	n/a	100.0%	n/a	100.0%	n/a
1	90.4%	0.05	91.9%	0.07	95.3%	0.05	95.3%	0.05
2	94.7%	0.03	92.3%	0.10	99.5%	0.06	95.4%	0.10
3	98.3%	0.06	88.8%	0.09	94.1%	0.12	96.3%	0.09
4	95.0%	0.05	81.8%	0.12	94.4%	0.11	91.7%	0.08
5	88.0%	0.13	85.1%	0.12	86.3%	0.11	85.2%	0.09
6	70.2%	0.16	72.4%	0.10	78.3%	0.13	74.9%	0.01
7	55.3%	0.18	57.9%	0.08	59.2%	0.11	64.2%	0.07
8	39.5%	0.18	43.7%	0.07	50.5%	0.15	53.2%	0.06
9	21.1%	0.17	33.4%	0.07	39.4%	0.17	38.9%	0.05
10	16.3%	0.15	15.5%	0.07	17.9%	0.09	25.0%	0.06

A. baumannii 4h Treatment

A.fum	CHD-FA Treatment Concentration (%)							
4h Tx	4.6		0.46		0.046		No Tx	
Day	Wound surface area	SD	Wound surface area	SD	Wound surface area	SD	Wound surface area	SD
0	100.0%	n/a	100.0%	n/a	100.0%	n/a	100.0%	n/a
1	100.0%	0.13	100.0%	0.09	94.5%	0.10	100.0%	n/a
2	96.5%	0.08	100.0%	0.09	96.4%	0.09	100.0%	n/a
3	86.4%	0.09	98.4%	0.05	88.8%	0.07	100.0%	n/a
4	83.6%	0.09	96.8%	0.10	87.2%	0.07	93.5%	n/a
5	77.7%	0.10	94.0%	0.09	88.0%	0.07	89.0%	n/a
6	57.8%	0.13	80.8%	0.08	76.1%	0.14	76.8%	n/a
7	48.6%	0.15	54.7%	0.07	67.6%	0.12	68.1%	n/a
8	32.6%	0.10	49.0%	0.10	56.3%	0.14	54.5%	n/a
9	20.9%	0.09	37.2%	0.05	34.7%	0.09	40.3%	n/a
10	9.6%	0.08	19.1%	0.08	29.4%	0.10	21.5%	n/a

A. fumigatus 4h Treatment

Section 7. Q6 Progress: 12/31/2013 through 03/31/2014.

2.2a. Establishing the wound infection in rats with carbapenen-resistant *Escherichia coli* strain DSP#182 (bla_{KPC}) (specific aim 2).

Our objective for the second quarter in the second year of this grant award was to complete the cutaneous wound infection models and assess CHD-FA treatment efficacy with agents listed in Specific Aims 2 of the statement of work. In this quarterly report, a cutaneous wound model in rats with the drug resistant (carbapenem) Gram negative bacteria *E. coli* and *Klebsiella pneumoniae* was established. Preliminary CHD-FA treatment efficacy data that included wound closure measurements and enumeration of microbial burden on wound sites were also collected on these pathogens.

As with the previously reported virulence assessment studies, 9 rats were randomized into three infection groups (3 rats per group). *E. coli* strain DSP#182 was inoculated in LB media and incubated at 37ºC with shaking overnight. Bacterial cells were washed, precipitated in sterile PBS, and diluted to $2x10^9$, $2x10^8$, and $2x10^7$ colony forming units (CFU) per ml for the infection. Two wounds were created on each rat, for a total of eighteen wounds. The process of anesthetizing the rats and creating the wounds was the same as described above in the section on establishing the wound model and in quarterly technical report Q5.01.2014. After the wound creation, rats from each challenge group were infected with 0.05 ml of the cell suspension with corresponding doses. The final infection doses for the rats were $1x10^8$, $1x10^7$, and $1x10^6$ CFU, respectively.

All the wounds infected with *E. coli* became purulent on days 1 or 2. The discharge of the wounds was observed at various infection doses throughout the 10-day experiment but was more pronounced in the higher infection doses. Initiation of scab formation was observed by day 1 and majority of the wounds had scabs by day 2 **(Fig.1)**. Wound closure in the rats was noticeably visible starting at day 5 at the two lower infection doses ($1x10^7$ and $1x10^6$ CFU) and by day 7 at the highest dose ($1 x10^8$). There were no observable signs of sepsis such as lethargy or recumbency as a result of infection in any of the rats. Daily digital wound measurement was taken with a Nikon 4x Stereomicroscope with FS-1 digital camera. Wound closure as percentage of wound surface area **(see Fig.2)** was discernible between the highest infection dose and the lower doses from days 6 through 8, however by the experimental endpoint at day 10 the majority of the untreated infected wounds were all similar in surface area measurements.

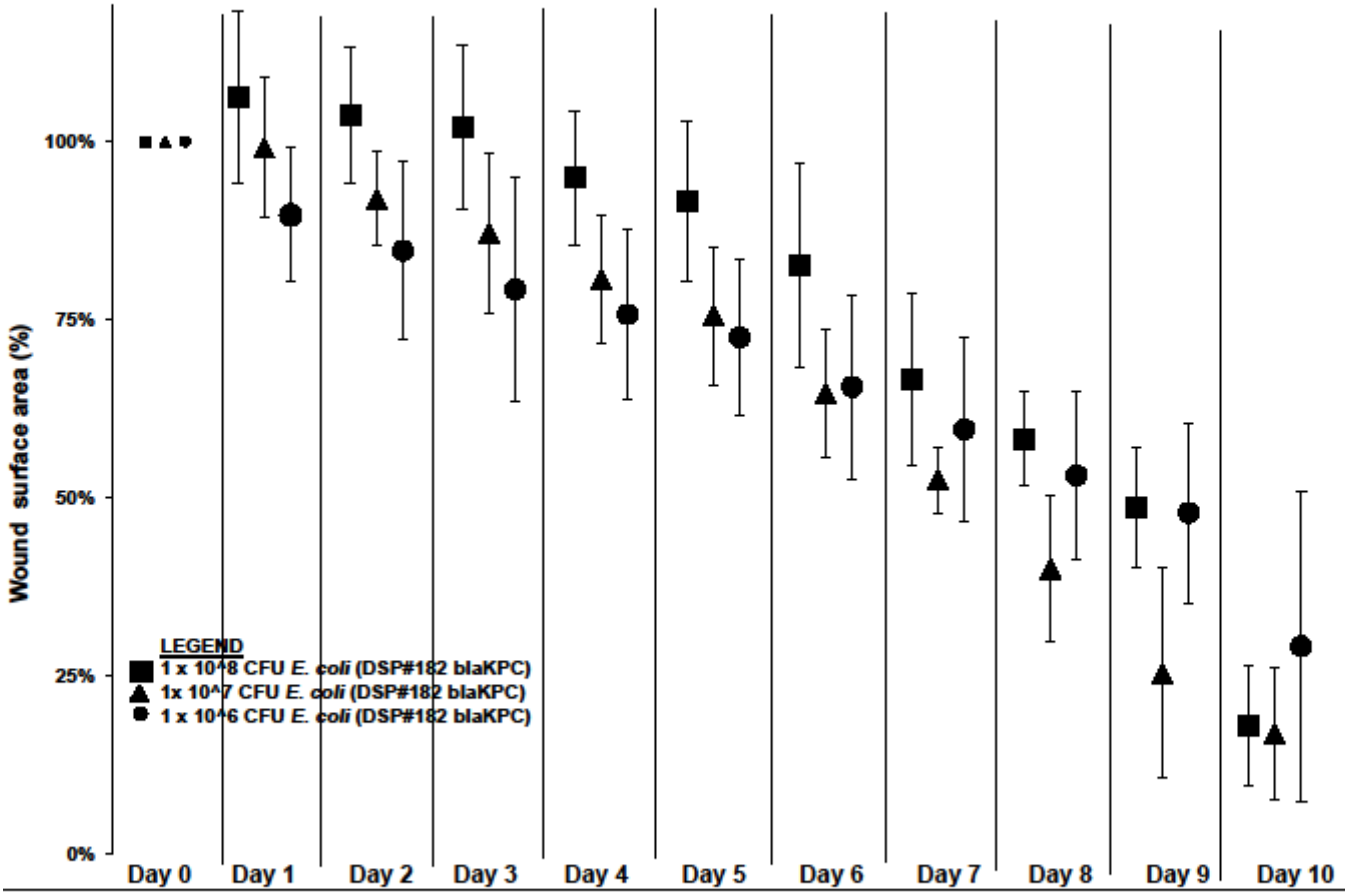
Conclusion

Based on the virulence study, a $1x10^8$ CFU infection dose was used for all downstream *E. coli* cutaneous wound infections

Figure 1. Cutaneous wounds in rats with *E. coli* strain DSP#182 bla_{KPC} infection.

Day	Rat #8 ($1x10^8$ CFU)	Rat #5 ($1x10^7$ CFU)	Rat #3 ($1x10^6$ CFU)
0			
1			
2			
10			

Figure 2. Wound surface area plot of wound infection model with *E. coli* strain DSP#182 bla$_{KPC}$.



Section 8. Establishing the wound infection in rats with *Klebsiella pneumoniae* strain BK#26594 (specific aim 2).

As with the *E. coli* virulence study, 9 rats were randomized into three infection groups (3 rats per group). *K. pneumonia* strain BK#26594 was inoculated in LB media and incubated at 37ºC with shaking overnight. Bacterial cells were washed, precipitated in sterile PBS, and diluted to 2x10^9, 2x10^8, and 2x10^7 CFU/ml for the infection. Two wounds were created on each rat, for a total of eighteen wounds. The process of anesthetizing the rats and creating the wounds was the same as described above in the section on establishing the wound model and in quarterly technical report Q5.01.2014. After the wound creation, rats from each challenge group were infected with 0.05 ml of the cell suspension with corresponding doses. The final infection doses for the rats were 1x10^8, 1x10^7, and 1x10^6 CFU, respectively.

All the wounds infected with *K. pneumonia* became purulent on days 1 and 2. The discharge of the wounds was observed at various infection doses throughout the 10-day experiment but was more pronounced in the higher infection doses. Biofilm formation near the wound bed/periphery was present in the majority starting on day 1 and was more pronounced at the higher infection doses. The wounds at the higher doses remained purulent and moist though the first 5 days. Scab formation did not develop until day 5 or later in the higher doses **(Fig.3)**. Wound closure in the rats was noticeably visible starting at day 7 at the two lower infection doses (1x10^7 and 1x10^6 CFU) and by day 8 at the highest dose (1 x10^8). There were no observable signs of sepsis such as lethargy or recumbency as a result of infection in any of the rats. Daily digital wound measurement was taken with a Nikon 4x Stereomicroscope with FS-1 digital camera. Wound closure as percentage of wound surface area

(see Fig.4) was discernible between the different infection doses starting on day 7. However scab formation during days 7 to the experimental endpoint at day 10 in the mid to lower infection doses prevented accurate wound measurement. The removal of the scab on day 10 revealed the wound closures were very similar among all infection doses.

Conclusion

Based on the virulence study and variability from the scab formation, a $1x10^8$ CFU infection dose will be used for all downstream *K. pneumoniae* cutaneous wound infections

Figure 3. Cutaneous wounds in rats with *K. pneumoniae* (BK#26594) infection.

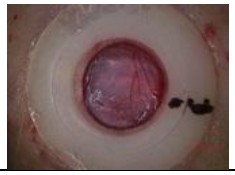
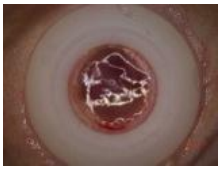
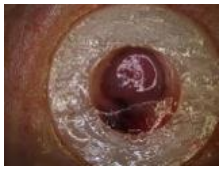
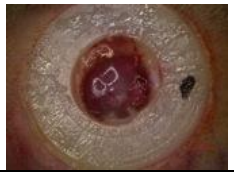
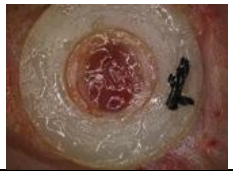
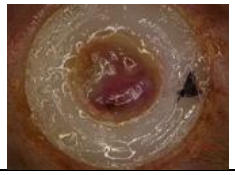
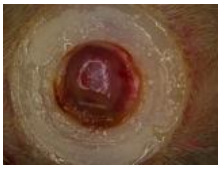
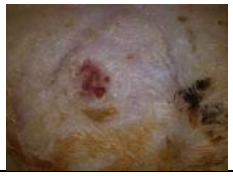
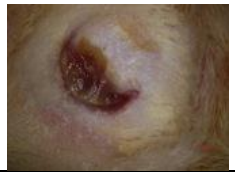
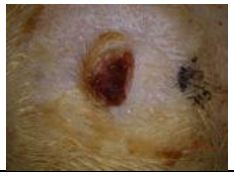
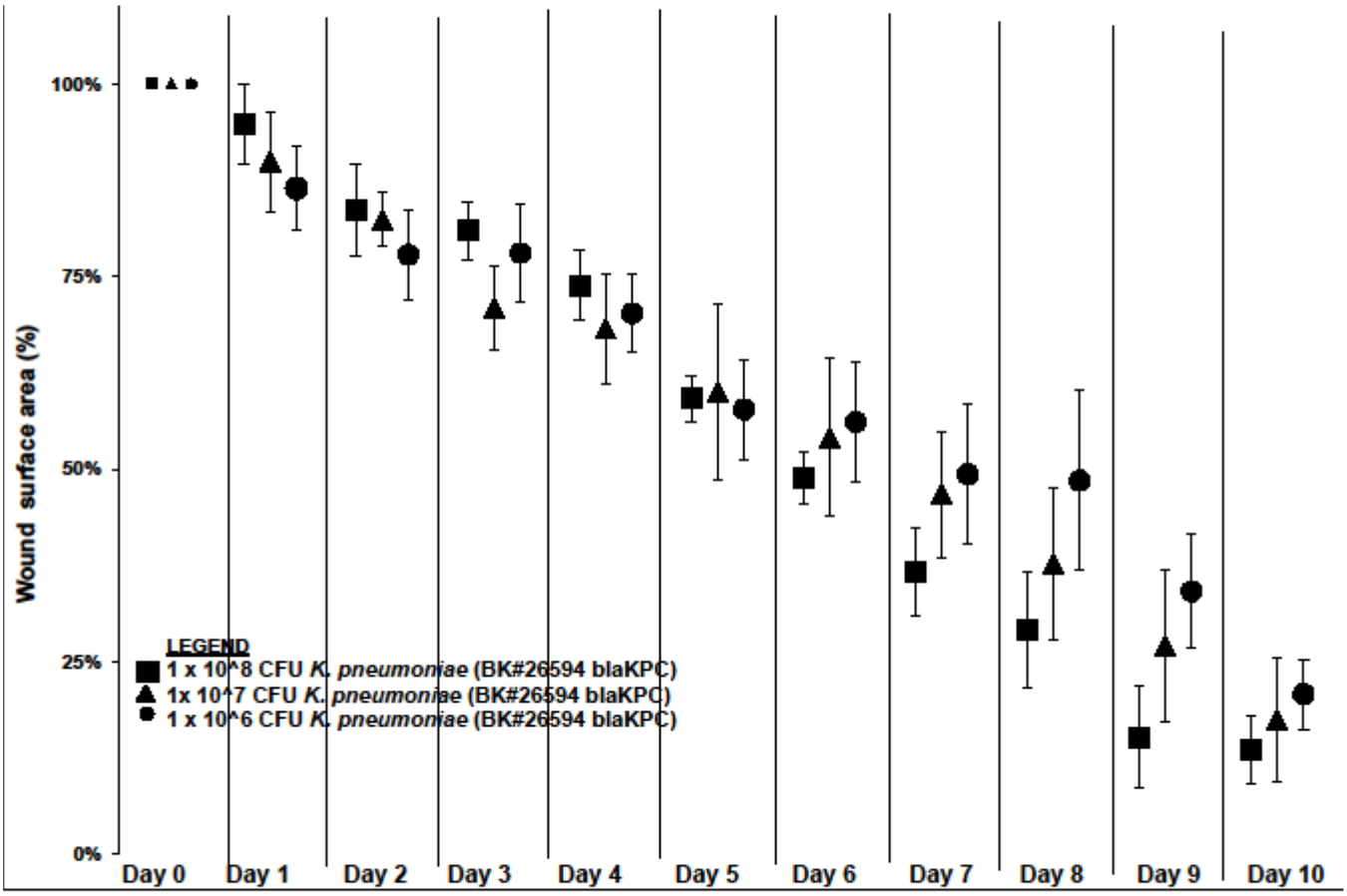
Day	Rat #8 ($1x10^8$ CFU)	Rat #5 ($1x10^7$ CFU)	Rat #3 ($1x10^6$ CFU)
0			
1			
2			
10			

Figure 4. Wound surface area plot of wound infection model with *K. pneumonia* strain BK#26594 bla$_{KPC}$.



Section 9. Evaluation of CHD-FA to treat cutaneous wounds infected with *Escherichia coli* strain DSP#182 (specific aim 2).

To assess the treatment efficacy of CHD-FA against cutaneous wounds infected with *E. coli*, 11 rats were randomized into three treatment groups (3 rats per group) and one untreated (sham) control and one antibiotic (Tetracycline; 5 ug/ml) treatment control group. E*. coli* DSP#182 was prepared at described in 2.2a. Wounds were created and infected with 0.05 ml of *E. coli* to a final infection dose of $1x10^8$ CFU*.* Rats were given daily treatments of CHD-FA at 4.6, 0.46 or 0.046% in 0.025 ml volumes starting at 4 hours post infection.

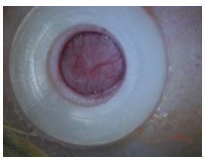
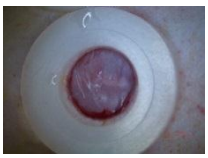
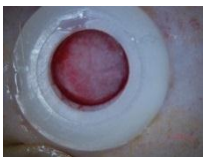
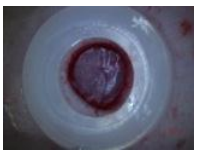
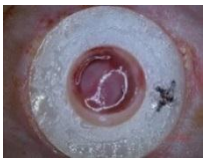
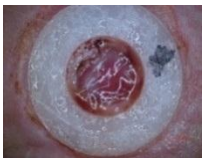
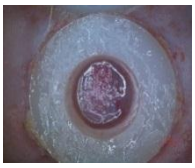
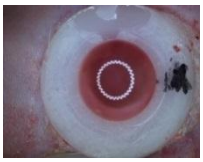
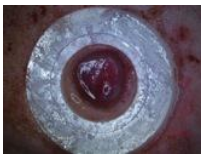
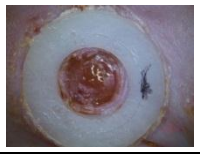
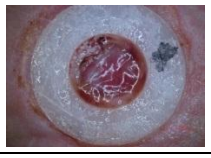
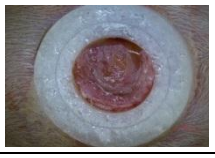
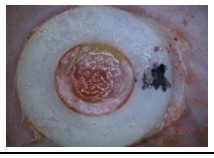
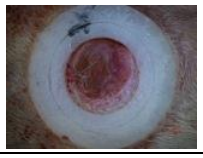
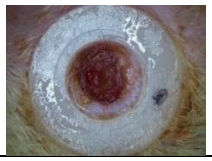
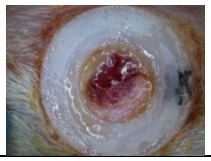
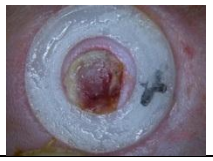
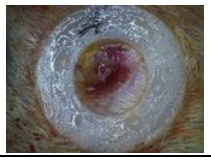
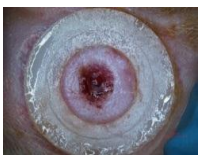
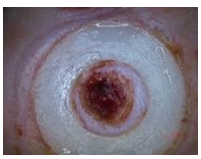
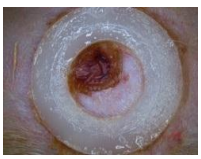
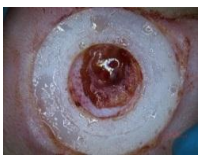
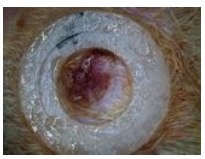
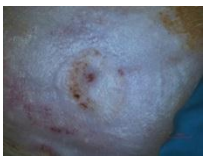
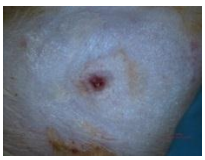
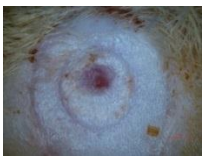
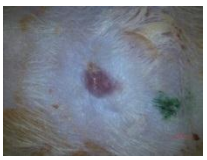
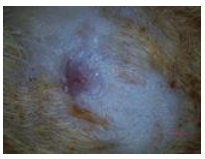
Improved wound closure was visually observed in the highest CHD-FA dose at 4.6% relative to the untreated control starting on day 7 **(Fig 5)**. The highest difference in wound closing occurred on days 8 and 9 of the infection. In our previous findings with other bacterial pathogens, the most improved wound closures were observed on days 5 to 7. As expected, both the CHD-FA treated and untreated wounds were significantly closed by the experimental endpoint at day 10. Purulent discharge was still observed in all of the rats; however, the duration and amount of discharge in the CHD-FA treated rats at 4.6% were less than the untreated controls by 1 to 3 days. Digital measurements of wound surface areas **(Table 1)** showed significant improvement in wound healing on days 6 to 9 with the highest (4.6%) CHD-FA treatment dose. The most prominent differences were on day 7 where the average wound surface areas were 17.6% in the high dose group (4.6%) in contrast to the untreated control at 42.5%. The lowest and mid CHD-FA (0.046%) treatment dose wound closure values were not significantly different from the untreated controls. However, less

purulent discharge was observed for the middle CHD-FA dose than the untreated controls. Bacterial burden analysis of the 4.6% CHD-FA treated wounds at the experimental endpoint showed a 2.3 log reduction relative to the untreated controls **(Table 2)**. The middle and lower CHD-FA doses did not significantly reduce the bacterial burden. Histopathologic evaluation of these wounds to better assess healing will be performed.

Conclusion

CHD-FA at the highest dose demonstrated significant reduction of the size and bacterial burden in the wound sites. These preliminary results are encouraging and these studies will be repeated with a focus on the earlier time points to assess bacterial burden. Furthermore, both histopathologic analyses and host gene expression profiling will be performed to better assess the cellular and molecular mechanisms during wound healing with CHD-FA.

Figure 5. Wound images of rats infected with *E. coli* (DSP#182) and treated with CHD-FA at 4h post infection.

Day	Rat #1 4.6% CHD-FA	Rat #4 0.46% CHD-FA	Rat #9 0.046% CHD-FA	Rat#10 Untreated	Rat#11 AB* Treated
0					
1					
2					
5					
7					
10					

*5.0 ug/ml Tetracycline

Section 10. Evaluation of CHD-FA to treat cutaneous wounds infected with *Klebsiella pneumoniae* strain BK#26594 bla$_{KPC}$ (specific aim 2).

To assess the treatment efficacy of CHD-FA against cutaneous wounds infected with *K. pneumonia* strain BK#26594 (bla$_{KPC}$), 11 rats were randomized into three treatment groups (3 rats per group), and an untreated (sham) and antibiotic (Colistin 20 ug/ml) treatment controls. *K. pneumonia* was prepared as previously described in 2.2b. Wounds were created and infected with 0.05 ml of bacteria to a final infection dose of 1x10^8 CFU. Rats were given daily treatments of antibiotic or CHD-FA at 4.6, 0.46 or 0.046% in 0.025 ml volumes starting at 4 h post infection. Improved wound closure was visually observed early in the infection (days 1 to 3) with the highest CHD-FA dose of 4.6%. This was due to the highly purulent discharge found in wounds that received the middle and low doses or CHD-FA or the untreated group. Digital measurements of the wounds corroborate these observations. The 4.6% CHD-FA had >10% improvement in wound closure relative to the untreated controls on days 1-3. The antibiotic control group (Colistin 20 ug/ml) also had improved wound closure early in the infection with >15% closure relative to the untreated wounds on day 2.

Scab formation did not occur until day 5 in the middle and low CHD-FA treated and untreated groups **(Fig 6)**. Purulent discharge was evident in all rats throughout the study, however the duration and amount of discharge were less in the CHD-FA treated rats at 4.6% relative to the untreated controls. The digital wound measurements showed the wound closure differential was marginally better (~10-12% improvement) with the highest CHD-FA treatment dose (4.6%) earlier in the infection (days 1 through 3) **(Table 1)**. Bacterial burden analysis of the wounds at the experimental endpoint revealed a modest 1.6 log reduction of CFU relative to the untreated control group **(Table 2)**. The other treatment doses did not significantly reduce the bacterial burden.

Conclusion

CHD-FA at the highest dose resulted in a reduction of the size and bacterial burden in the wound sites. Most noticeably, infected wound that were given the highest CHD-FA dose formed scabs earlier and less purulent discharge than the untreated group. The antibiotic (20 ug/ml Colistin) treated wounds behaved similarly. These preliminary results are encouraging and these studies will be repeated with a focus on the earlier time points to assess bacterial burden. Furthermore, both histopathologic analyses and host gene expression profiling will be performed to better assess the cellular and molecular mechanisms during wound healing with CHD-FA.

Figure 6. Wound images of rats infected with *K. pneumoniae* (BK#26594) and treated with CHD-FA at 4h post infection.

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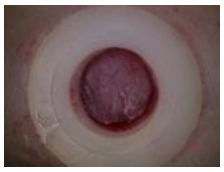
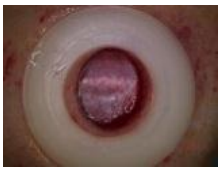
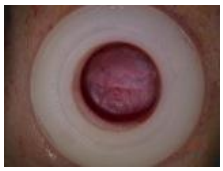
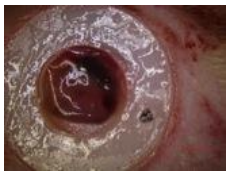
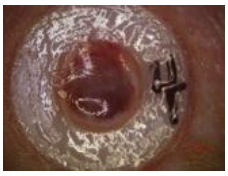
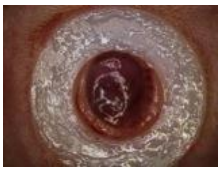
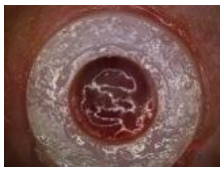
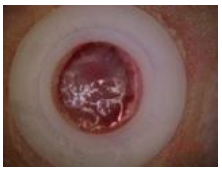
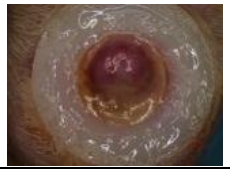
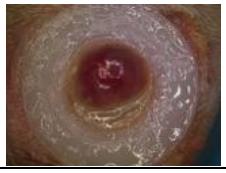
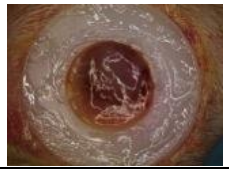
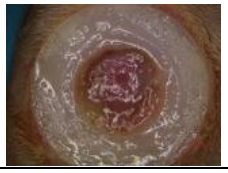
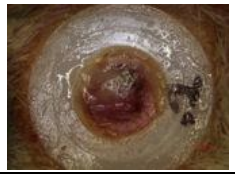
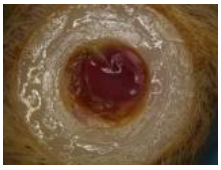
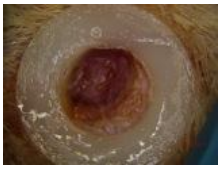
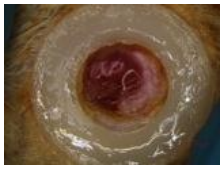
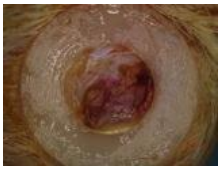
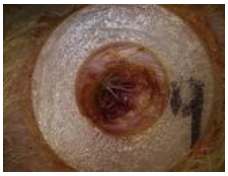
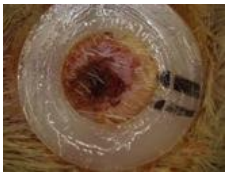
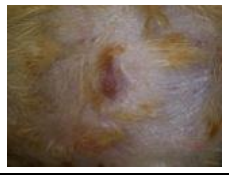
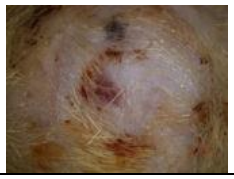
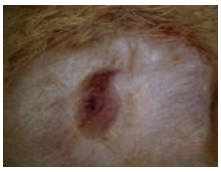
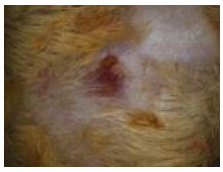
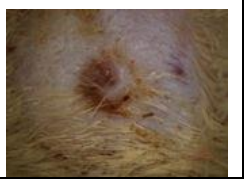
0					
1					
2					
5					
7					
10					

*20.0 ug/ml Colistin

Table 1. Wound closure analysis of Rats infected with *E. coli* and *K. pneumonia* treated with CHD-FA.

Ec KPC	CHD-FA Treatment Concentration (%)									
4h Tx	4.6		0.46		0.046		No Tx		AB Tx	
Day	Wound surface area	SD	Wound surface area	SD	Wound surface area	SD	Wound surface area	SD	Wound surface area	SD
0	100.0%	n/a	100.0%	n/a	100.0%	n/a	100.0%	n/a	100.0%	n/a
1	88.8%	0.04	85.4%	0.04	89.0%	0.05	82.2%	n/a	77.9%	n/a
2	79.1%	0.04	89.1%	0.06	87.6%	0.09	70.6%	n/a	84.2%	n/a
3	78.6%	0.05	79.2%	0.11	79.8%	0.02	73.0%	n/a	69.6%	n/a
4	76.2%	0.08	66.9%	0.14	66.9%	0.07	66.0%	n/a	55.3%	n/a
5	62.7%	0.10	69.7%	0.06	70.0%	0.05	61.5%	n/a	46.1%	n/a
6	37.9%	0.13	53.1%	0.11	54.9%	0.09	51.5%	n/a	34.0%	n/a
7	17.6%	0.02	42.5%	0.08	42.5%	0.13	33.2%	n/a	28.0%	n/a
8	12.3%	0.02	33.5%	0.09	36.4%	0.14	27.1%	n/a	30.5%	n/a
9	4.9%	0.03	23.7%	0.09	31.1%	0.09	20.6%	n/a	21.7%	n/a
10	1.4%	0.01	14.4%	0.06	18.5%	0.04	17.6%	n/a	6.9%	n/a

E. coli DSP182 blaKPC 4h Treatment

Kp KPC	CHD-FA Treatment Concentration (%)									
4h Tx	4.6		0.46		0.046		No Tx		AB Tx	
Day	Wound surface area	SD	Wound surface area	SD	Wound surface area	SD	Wound surface area	SD	Wound surface area	SD
0	100.0%	n/a	100.0%	n/a	100.0%	n/a	100.0%	n/a	100.0%	n/a
1	87.3%	0.06	87.3%	0.07	98.9%	0.06	98.2%	n/a	97.1%	n/a
2	78.5%	0.06	89.0%	0.04	89.5%	0.06	89.4%	n/a	73.4%	n/a
3	64.0%	0.06	80.6%	0.07	82.6%	0.08	73.0%	n/a	47.5%	n/a
4	55.0%	0.06	72.6%	0.07	68.3%	0.08	61.9%	n/a	45.8%	n/a
5	52.6%	0.05	57.9%	0.02	59.1%	0.07	54.8%	n/a	41.6%	n/a
6	42.0%	0.02	46.7%	0.09	50.0%	0.09	44.3%	n/a	19.2%	n/a
7	27.9%	0.02	33.2%	0.05	31.8%	0.06	30.0%	n/a	13.0%	n/a
8	22.2%	0.03	23.4%	0.05	22.3%	0.04	17.2%	n/a	6.0%	n/a
9	16.0%	0.04	20.9%	0.03	23.6%	0.09	17.7%	n/a	15.4%	n/a
10	7.5%	0.02	9.1%	0.03	14.6%	0.04	5.5%	n/a	12.5%	n/a

K. pneumoniae BK26594 blaKPC 4h Treatment

Table 2. Bacterial burdens of cutaneous wounds infected with *E. coli* or *K. penumoniae* at experimental endpoint (10 days, post infection)

Treatment	Avg. Log CFU	Range	Log fold changes	Study
No Tx Control	4.8	n/a	n/a	*E. coli* 4h Tx
4.60%	2.5	1.7-2.9	2.3	
0.46%	4.4	2.0-5.8	0.4	
0.046%	4.7	4.1-4.7	0.1	
AB control	4.1	n/a	0.7	

Treatment	Avg. Log CFU	Range	Log fold changes	Study
No Tx Control	5.9	n/a	n/a	*K.pneumoniae* 4h Tx
4.60%	4.3	3.5-5.4	1.6	
0.46%	5.2	4.4-5.6	0.7	
0.046%	5.1	4.9-5.4	0.8	
AB control	5.7	6.2-6.9	0.2	

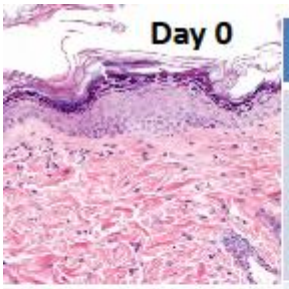
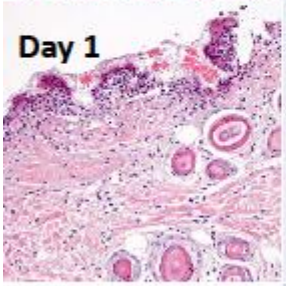
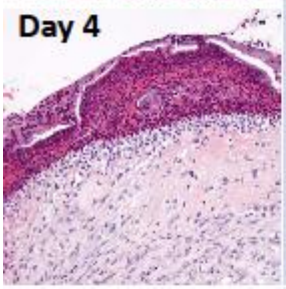
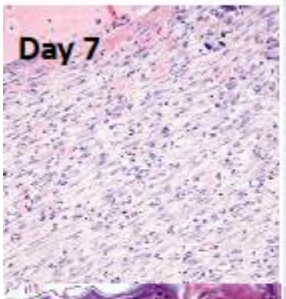
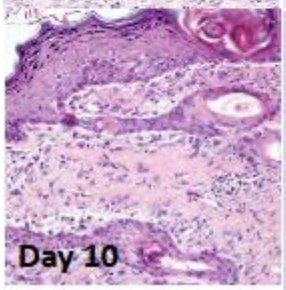
Section 11. Histological evaluation of uninfected wounds.

Histological evaluation on the uninfected wounds from day 0 to day 10 was performed. The uninfected wounds were used first to establish the scoring parameters during the natural healing process throughout the 10 day trial. Wounds were prepared as previously described without the addition of infectious agents. The wounds were aseptically excised daily starting at the initial wound creation through the experimental endpoint on day 10. Each wound was fixed in neutral buffered formalin, sectioned and stained with hematoxylin and eosin (H&E). The wounds were microscopically evaluated for the presence of neutrophils and macrophages to assess inflammation, the initiation of healing by the presence of fibroblasts and indicators of angiogenesis and lastly epithelialization to complete the remodeling of the wound site. The detailed description of the scoring system for histological analysis is listed on **Table 3**. As expected, the initial wound creation on day 0 **(Fig 7.)**, no evidence of inflammation was presence. On day 1, the onset of inflammation on the wound site is observed and the initiation of healing is evident by the presence of neutrophils. By day 4, the inflammation of the wound site is still present but increased presence of fibroblasts and angiogenesis strongly indicates the remodeling of the wound site. On Day 7 epithelialization is increasing and remodeling of the wound site is evident. By day 10, the remodeling is completed on wound site visually, but there is still presence of fibroblasts and angiogenesis microscopically.

Table 3. Scoring summary for histological analysis

Score	Neutrophils	Macrophages	Fibroblasts	Angiogenesis	Epithelialization
0	No Neutrophils	No macrophages	No fibroblasts	None	No epithelialization
1	Scattered Neutrophils	Scattered macrophages	Poorly organized fibroblasts	Rare immature vessels	Early epithelialization at edges of wound
2	Multifocal aggregates of Neutrophils	Clusters of macrophages	Moderately organized fibroblasts	Moderate numbers of immature vessels	Complete epithelialization, but not all layers
3	Diffuse infiltrates of neutrophils	Diffuse infiltrates of macrophages	Well organized fibroblasts	Many immature vessels	Complete epithelialization

Figure 7. Histological evaluation of uninfected wounds.

	Neutrophils	Macrophages	Fibroblasts	Angiogenesis	Epithelization
	0	0	0	0	0
	2	0	0	0	0
	2	1	3	3	1
	1	3	3	3	2
	1	3	2	2	3

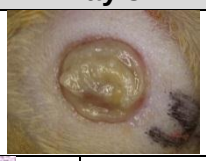
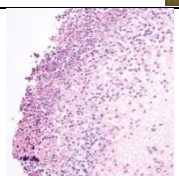
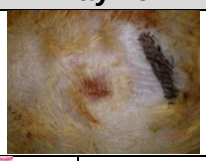
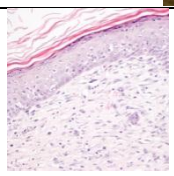
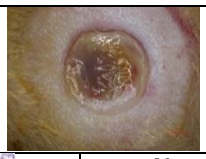
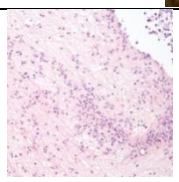
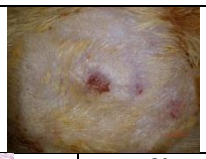
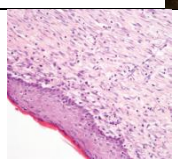
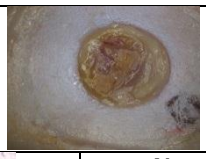
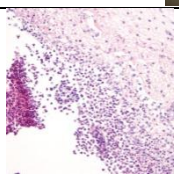
Conclusion

Histopathological analysis provides practical measure to assess wound healing and evaluate treatment efficacy of CHD-FA in the wound infection models.

Section 12. Histopathological analysis of wounds from rats infected with MRSA Xen31 and treated with CHD-FA.

Histopathogical analysis was performed on cutaneous wounds of rats infected with MRSA (Xen31) treated with CHD-FA. Rats were randomized into three treatment groups (3 rats per group) and one untreated (sham) control and one antibiotic treatment control group. MRSA was prepared at described in quarterly report Q3. Wounds were created and infected with 0.05 ml of MRSA to a final infection dose of $1x10^8$ CFU. Rats were given daily treatments of CHD-FA at 4.6 or 0.46% in 0.025 ml volumes starting at 4 hours post infection. On days 3 and 10 wounds were aseptically excised. Each wound was fixed in neutral buffered formalin, sectioned and stained with hematoxylin and eosin (H&E). The wounds were microscopically evaluated for the presence of neutrophils and macrophages to assess inflammation, the initiation of healing by the presence of fibroblasts and indicators of angiogenesis and lastly epithelialization to complete the remodeling of the wound site. The detailed description of the scoring system for histological analysis is listed on **Table 3**. The histopathologic evaluation of the CHD-FA and untreated wounds samples from day 3 showed higher scores in both macrophage and fibroblast categories in the 4.6% CHD-FA treated wound (**Fig. 8**). This is indicative of earlier wound healing and remodeling. On Day 10, epithelialization scores for the wounds treated with CHD-FA was higher than the untreated wound. Conversely, the neutrophil score was higher in the untreated wound. The decrease in cellular inflammation and increase in epithelialization suggest wound healing is significantly improved with CHD-FA treatment.

Figure 8. Histopathological analysis of wounds from rats infected with MRSA Xen31 and treated with CHD-FA at 4h post infection.

<table>
<tr><th></th><th>Day 3</th><th>Day 10</th></tr>
<tr><td>4.6%
(CHD-FA)</td><td>


<table>
<tr><td>Neutrophils</td><td>3</td></tr>
<tr><td>Macrophages</td><td>3</td></tr>
<tr><td>Fibroblasts</td><td>2</td></tr>
<tr><td>Angiogenesis</td><td>2</td></tr>
<tr><td>Epithelization</td><td>0</td></tr>
</table>
The epidermis is completely effaced by large mats of fibrillar eosinophilic material mixed with abundant degenerate neutrophils and large colonies of coccoid bacteria. The underlying dermis is expanded by reactive fibroblasts, immature blood vessels and many neutrophils and macrophages. Inflammatory cells infiltrate the underlying skeletal muscle.
</td><td>


<table>
<tr><td>Neutrophils</td><td>1</td></tr>
<tr><td>Macrophages</td><td>1</td></tr>
<tr><td>Fibroblasts</td><td>3</td></tr>
<tr><td>Angiogenesis</td><td>3</td></tr>
<tr><td>Epithelization</td><td>3</td></tr>
</table>
From biopsy center, the dermis is expanded by scattered neutrophils and macrophages mixed with abundant well-organized reactive fibroblasts and immature blood vessels. There is a focal aggregate of basophilic granular material (mineral) surrounded by multinucleate giant cells in the dermis. Inflammatory infiltrates invade the underlying skeletal muscle.
</td></tr>
<tr><td>0.46%
(CHD-FA)</td><td>


<table>
<tr><td>Neutrophils</td><td>3</td></tr>
<tr><td>Macrophages</td><td>2</td></tr>
<tr><td>Fibroblasts</td><td>1</td></tr>
<tr><td>Angiogenesis</td><td>2</td></tr>
<tr><td>Epithelization</td><td>0</td></tr>
</table>
The epidermis is missing from the center of the section, and the exposed dermis is covered by a partially detached crust composed of abundant fibrin and degenerate neutrophils, with many superficial colonies of coccoid bacteria. The dermis is expanded by abundant clear space (edema) with many reactive fibroblasts and immature blood vessels. Inflammation extends to the underlying skeletal muscle and adipose tissue.
</td><td>


<table>
<tr><td>Neutrophils</td><td>1</td></tr>
<tr><td>Macrophages</td><td>2</td></tr>
<tr><td>Fibroblasts</td><td>3</td></tr>
<tr><td>Angiogenesis</td><td>2</td></tr>
<tr><td>Epithelization</td><td>3</td></tr>
</table>
At the center of the section, the dermis is expanded by abundant reactive fibroblasts, arranged parallel to the skin surface, and immature blood vessels, arranged perpendicular to the surface (organized granulation tissue). There are scattered neutrophils and macrophages in the superficial dermis, with infiltrates of lymphocytes in the deeper dermis. Scattered clusters of multinucleate giant cells and neutrophils surround hair fragments.
</td></tr>
<tr><td>Untreated
(Saline)</td><td>


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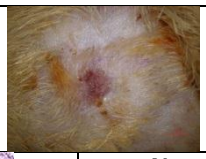
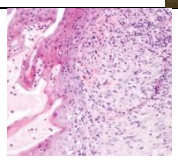
<tr><td>Macrophages</td><td>2</td></tr>
<tr><td>Fibroblasts</td><td>1</td></tr>
<tr><td>Angiogenesis</td><td>2</td></tr>
<tr><td>Epithelization</td><td>0</td></tr>
</table>
The epidermis is missing from the center of the section and the dermis is covered by a thick mat of fibrin, bacterial colonies and degenerate neutrophils. The dermis is edematous and expanded by dense infiltrates of neutrophils and macrophages on a background of loosely arranged fibroblasts and immature blood vessels. The inflammation extends to the underlying skeletal muscle and adipose tissue.
</td><td>


<table>
<tr><td>Neutrophils</td><td>2</td></tr>
<tr><td>Macrophages</td><td>2</td></tr>
<tr><td>Fibroblasts</td><td>3</td></tr>
<tr><td>Angiogenesis</td><td>2</td></tr>
<tr><td>Epithelization</td><td>2</td></tr>
</table>
At the center of the section, the stratum corneum is expanded by lakes of eosinophilic granular material (serum) and moderate numbers of neutrophils. The underlying dermis contains scattered aggregates of neutrophils and macrophages on a background of moderately organized plump fibroblasts and few immature blood vessels. Inflammation extends to the underlying skeletal muscle.
</td></tr>
</table>

Q8 Progress: **07/01/2014 through 09/30/2014.**

Section 13. Expression profiling of host wound healing genes in cutaneous wounds infected with MRSA strain Xen31 and Pseudomonas strain Xen5 (specific aim 2).

To better assess the treatment efficacy of CHD-FA in the early stages of infection against cutaneous wounds infected with MRSA and *P. aeruginosa*, cellular and molecular mechanisms were assessed by host gene expression profiling. Tissue healing and remodeling profiling was evaluated using a commercial PCR array (The Rat Wound Healing RT² Profiler™ PCR Array, Cat No. 330231, Qiagen). This kit assayed 84 genes essential to wound healing process, including extracellular matrix (ECM) remodeling factors, inflammatory cytokines and chemokines, as well as growth factors and major signaling molecules. Wound healing progresses via three overlapping phases: inflammation, granulation and tissue remodeling. After cutaneous injury, a blood clot forms and inflammatory cells infiltrate the wound, secreting cytokines and growth factors to promote the inflammation phase. During the granulation phase, fibroblasts and other cells differentiate into myofibroblasts, which deposit ECM proteins. Simultaneously, angiogenesis occurs, in which keratinocytes proliferate and migrate to close the wound. In the final tissue remodeling phase, apoptosis eliminates myofibroblasts and extraneous blood vessels, and the ECM is remodeled to resemble the original tissue. Dysregulation of this last tissue remodeling phase leads to fibrosis. The acute inflammatory phases of wounds are characterized by changes in gene expression during healing. Genes were also clustered on the basis of their functional annotation, linking them to inflammation, angiogenesis, reactive oxygen species or ECM categories.

During this quarter host gene expression profiling studies of MRSA and *P. aeruginosa* infected wounds were performed. This involved the collection of the wound samples (tissues), RNA extraction from the tissue, cDNA synthesis and performing real time PCR on the cDNA sample using a PCR array plate. The wound samples (tissue) were obtained via a biopsy punch during the corresponding time points from the MRSA and *P. aeruginosa* infection models and were stored frozen at -80°C. For RNA extraction, the samples were thawed and sectioned as to obtain between 10 to 30mg of the tissueas recommended by the manufacturer.
The Qiagen RNeasy Fibrous Tissue Mini Kit (Catalog # 74704) was used to isolate total RNA by following the recommended protocol. The concentration and purity of RNA obtained was determined by measuring the absorbance by using a Nanodrop spectrophotometer. After calculating the final concentration of RNA in the tissue sample, 0.5 ug of total RNA was used to proceed to cDNA synthesis by using the Qiagen RT^2 First Strand Kit (Catalog # 330401).

The cDNA synthesis was carried out by eliminating the genomic DNA in the RNA sample then followed by addition of the reverse transcription mix. (RT² Profiler PCR Array handbook – Protocol Pg 27-28). The cDNA obtained was used to perform real time PCR assay using the Rat wound Healing PCR array plates from Qiagen (Qiagen RT² Profiler PCR Array; Cat No. 330231). The high-quality primer design and RT^2 SYBR® Green qPCR Mastermix formulation enabled the PCR array to amplify and monitor the expression of 84 genes related to a wound healing, and five housekeeping genes simultaneously under uniform cycling conditions. Each array included control elements for data normalization, a Genomic DNA Control (GDC) assay, triplicate Reverse Transcription Controls (RTC), and triplicate Positive PCR Controls (PPC).

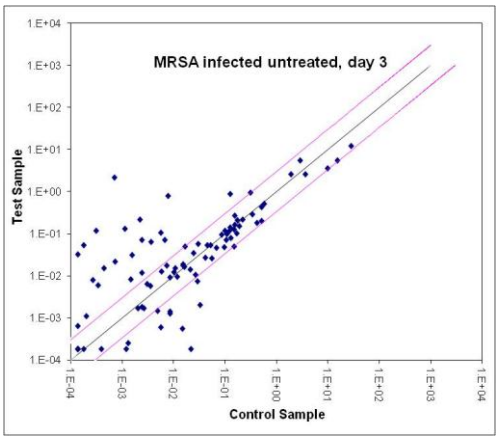
Qiagen RT^2 SYBR® Green ROX qPCR Mastermix (Cat. No. 330522) was used to set up real time assay with the prepared cDNA sample. The cDNA sample was gently mixed with ready to use mastermix and aliquoted into equal volumes of 0.025ml into each well of the 96 well plate, and real-time PCR was performed on an Mx3005P qPCR instrument (Agilent). Real-time PCR array data were analyzed by using the RT^2 profiler PCR array data analysis online software (Qiagen). Data analysis is

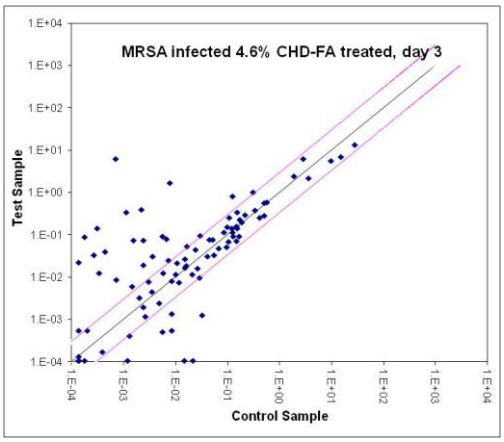
based on the ΔΔCT method with normalization of the raw data to either housekeeping gene provided in the Array kit.

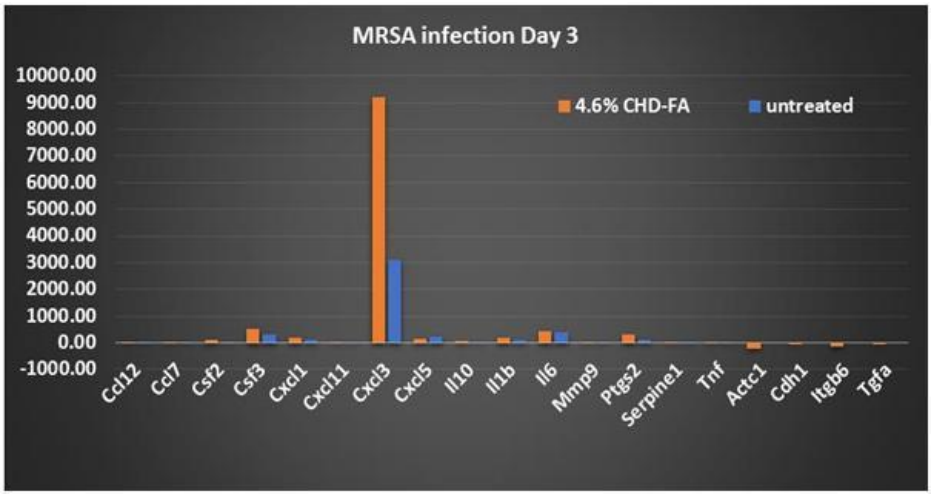
MRSA infected rat wound model. Of the 84 key genes central to wound healing, there was a marked increase of 5-fold or greater in 15 genes, CCL12, CCL7, CSF2, CSF3, CXCL1, CXCL11, CXCL3, CXCL5, IL10, IL1b, IL6, MMP9, PTGS2, SERPINE1, and TNF; and 4 genes, ACTC1, CDH1, ITGB6, and TGFA, had decreased expression by 5-fold or greater in infected wounds, compared to baseline normal skin (skin punctures collected from wound creation served as a baseline control). At day 3, wounds from both 4.6% CHD-FA treatment and sham control groups shared similar expression profiles, except the expression of CSF2 (granulocyte-macrophage-colony stimulating factor, GM-CSF), CXCL3 (inflammatory chemokine), IL10 (anti-inflammatory factor), and PTGS2 (signal transduction) was up-regulated by an additional 3-fold in CHD-FA treated wound compared to the sham control (**Figure 1**), indicating more active inflammation, anti-inflammation interaction towards accelerated wound healing in CHD-FA treated wounds. By day 10, while the expression level of most of these genes returned to normal, some genes were still expressed at 5-fold or greater level compared to normal skin. Such persistent overexpression was observed with 4 genes (CCL12, CXCL11, CXCL3, and CXCL5) in CHD-FA treated wound, in contrast to 7 genes (CCL12, CCL7, CXCL11, CXCL3, CXCL5, IL6, and MMP9) in the untreated wound. CHD-FA treated wound at day 10 is improved relative to the untreated as indicated by the absence of the overexpression of pro-inflammatory markers CCL7, IL-6 and MMP9 **(Figure 3, 4)**.

The day 6 time point for expression profiling was selected based on the differences observed in histopathological data and wound measurements between the CHD-FA treated and untreated wounds. Interestingly, the same pattern of gene expression at day 6 was observed as with the day 3 data as shown in **(Figure 2)**

Figure.1 Day 3 post infection wound healing gene expression profiling Untreated vs. 4.6% CHD-FA treatment in MRSA infected wounds

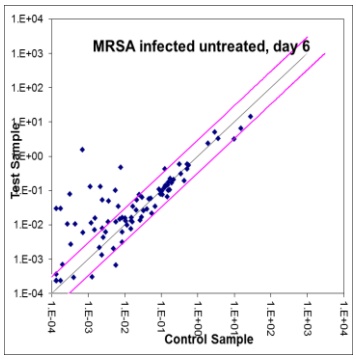


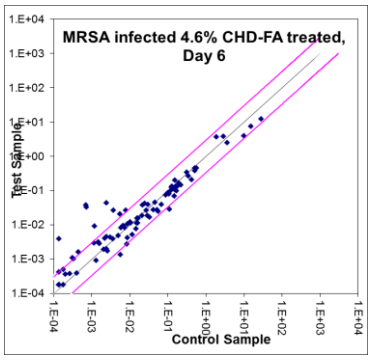


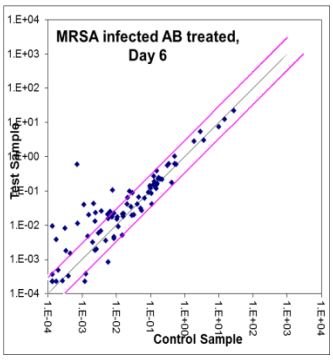


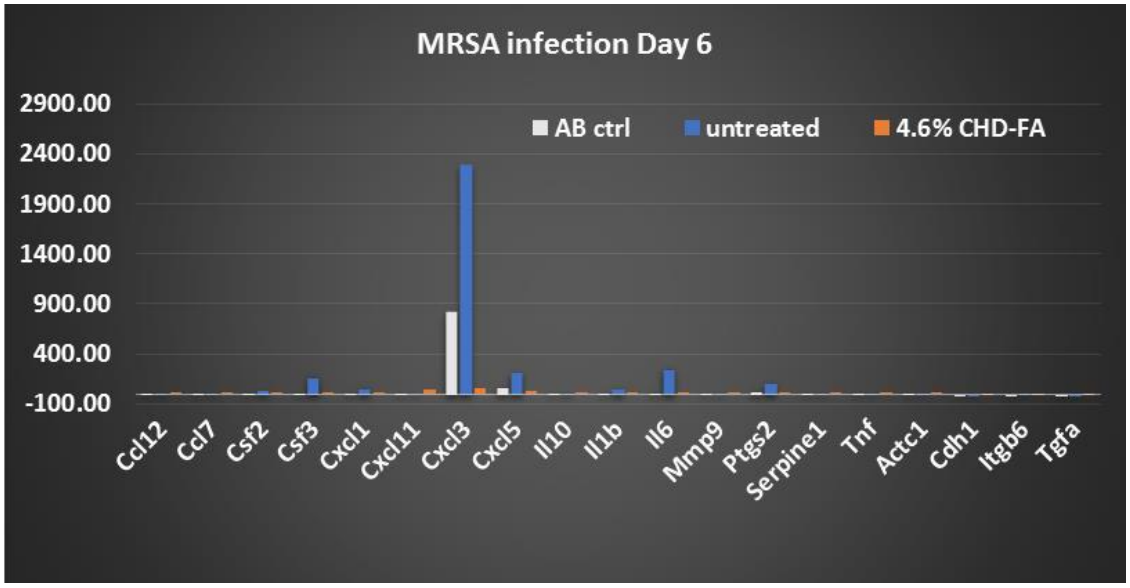
MRSA Infection Day 3 vs. Day 0					
Increased expression			Decreased expression		
Fold change			Fold change		
Gene name	Untreated	4.6% CHD-FA	Gene name	Untreated	4.6% CHD-FA
Ccl12	29.71	30.93	Actc1	-113.54	-197.95
Ccl7	19.67	16.63	Cdh1	-15.64	-25.44
Csf2	30.44	126.76	Itgb6	-25.40	-135.20
Csf3	305.07	505.30	Tgfa	-5.91	-15.28
Cxcl1	100.64	179.89			
Cxcl11	30.02	11.84			
Cxcl3	3110.65	9159.16			
Cxcl5	246.08	164.39			
Il10	35.33	89.95			
Il1b	103.82	213.19			
Il6	388.83	461.76			
Mmp9	17.85	8.49			
Ptgs2	118.85	306.77			
Serpine1	10.88	11.97			
Tnf	18.16	38.35			

Figure 2. Day 6 post infection wound healing gene expression profiling Untreated vs. 4.6% CHD-FA treatment in MRSA infected wounds



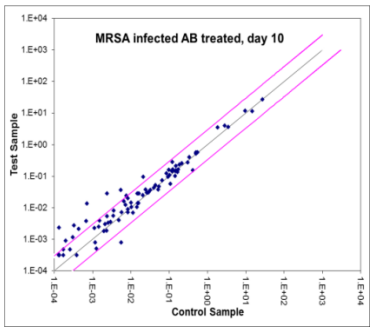


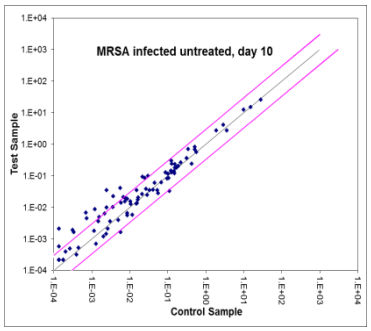


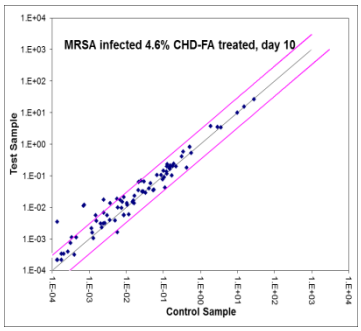


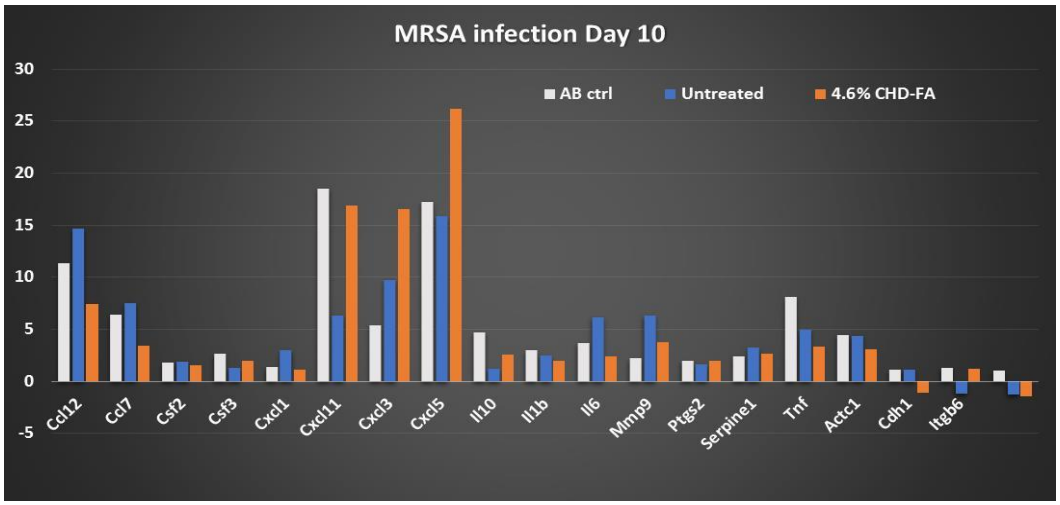
MRSA Infection Day 6 vs. Day 0							
Increased expression				Decreased expression			
Fold change				Fold change			
Gene name	AB ctrl	Untreated	4.6% CHD-FA	Gene name	AB ctrl	Untreated	4.6% CHD-FA
Ccl12	9.32	22.67	18.32	Actc1	2.95	2.55	1.77
Ccl7	3.77	18.74	3.76	Cdh1	-1.65	-1.25	-1.93
Csf2	0.87	40.17	2.88	Itgb6	-1.30	1.70	-1.47
Csf3	21.27	174.01	0.90	Tgfa	-1.91	-1.36	-2.00
Cxcl1	19.17	60.25	1.90				
Cxcl11	15.20	8.33	45.57				
Cxcl3	832.32	2292.97	54.38				
Cxcl5	67.70	226.44	29.75				
Il10	3.27	24.64	3.64				
Il1b	13.51	61.31	3.41				
Il6	25.83	260.11	3.54				
Mmp9	7.09	13.62	7.36				
Ptgs2	34.80	115.60	2.61				
Serpine1	3.71	5.29	1.45				
Tnf	5.25	8.42	3.08				

Figure 3. Day 10 post infection wound healing gene expression profiling Untreated vs. 4.6% CHD-FA treatment in MRSA infected wounds



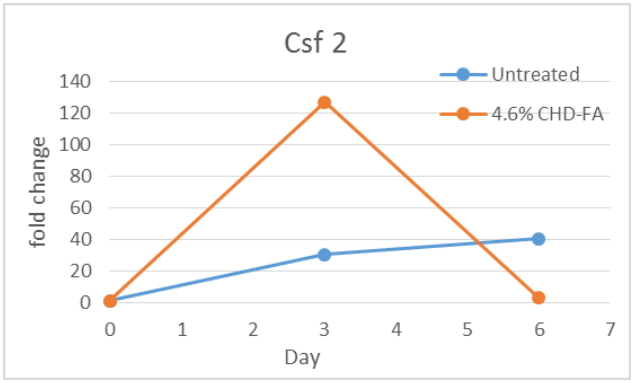


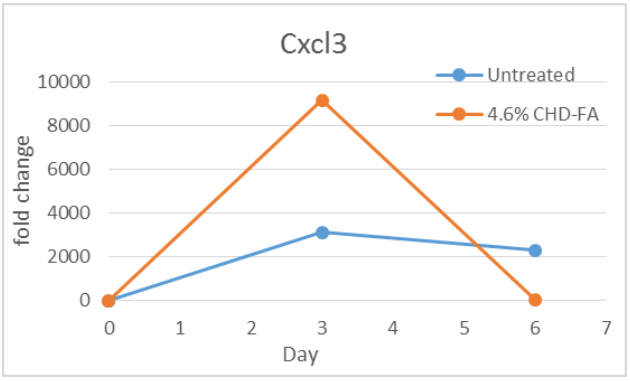


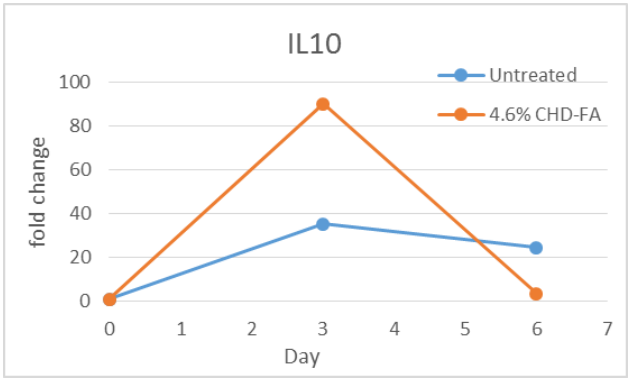


MRSA Infection Day 10 vs. Day 0							
Increased expression				Decreased expression			
Fold change				Fold change			
Gene name	AB ctrl	Untreated	4.6% CHD-FA	Gene name	AB ctrl	Untreated	4.6% CHD-FA
Ccl12	11.37	14.67	7.41	Actc1	4.43	4.36	3.03
Ccl7	6.42	7.46	3.42	Cdh1	1.09	1.09	-1.12
Csf2	1.76	1.89	1.50	Itgb6	1.28	-1.20	1.23
Csf3	2.63	1.24	1.96	Tgfa	1.02	-1.28	-1.44
Cxcl1	1.38	2.98	1.07				
Cxcl11	18.53	6.32	16.84				
Cxcl3	5.38	9.75	16.55				
Cxcl5	17.23	15.83	26.15				
Il10	4.71	1.18	2.55				
Il1b	2.99	2.48	1.94				
Il6	3.67	6.17	2.43				
Mmp9	2.19	6.32	3.74				
Ptgs2	1.98	1.62	1.94				
Serpine1	2.39	3.19	2.67				
Tnf	8.09	4.91	3.36				

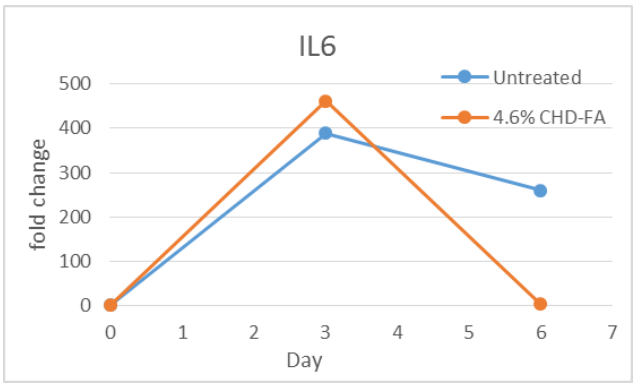
Figure 4. Temporal changes of individual gene targets for untreated and CHD-FA treated in MRSA infected wounds.







CSF2 (granulocyte-macrophage-colony stimulating factor, GM-CSF), CXCL3 (inflammatory chemokine), IL10 (anti-inflammatory factor) was up-regulated by an additional 3-fold in CHD-FA treated wound compared to the sham control, indicating more active inflammation, anti-inflammation interaction towards accelerated wound healing in CHD-FA treated wounds.

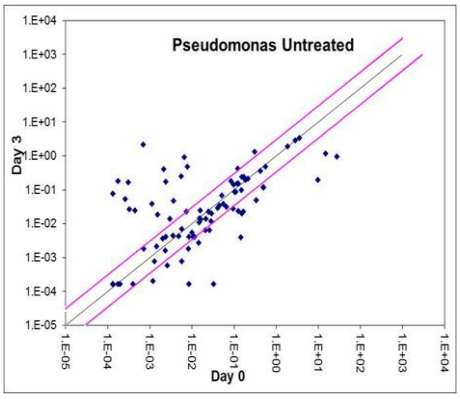


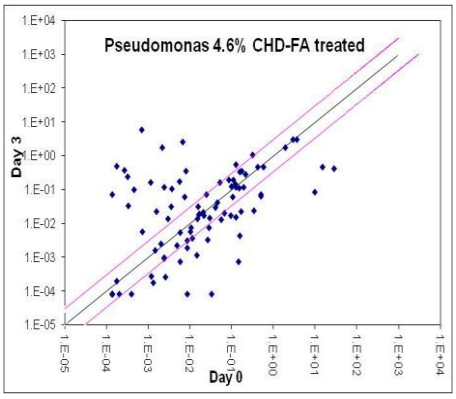
The key biomarker of impaired wound healing IL6 was constantly overexpressed through day 3 to day 6 in the untreated wound, the significantly reduced (74-fold) expression level in the CHD-FA treated wound manifested accelerated and better controlled wound healing.

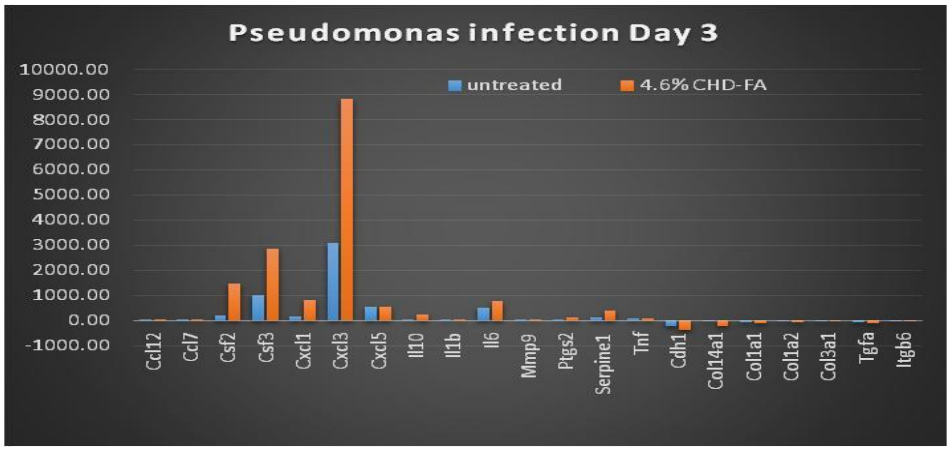
***P. aeruginosa* infected rat wound model**. The expression profile of P. aeruginosa infected wounds at day 3 was very similar but with more drastic changes in comparison to MRSA infected wounds. Genes that significantly overexpressed were almost identical with those described above for MRSA infected wounds at the same time point. Whereas, more genes with decreased expression were found in *P. aeruginosa* infected wounds compared to that infected with MRSA. These genes are mainly involved in extracellular matrix component (COL14a1, COL1a1, and COL1a2) and cellular adhesion (CDH1 and ITGB6), indicating a more profound tissue damage caused by *P. aeruginosa* compared to MRSA at the studied infection doses. As expected, the expression changes at day 3 were generally greater in the CHD-FA treated wound than in the sham control **(Figure 5)**. Namely, growth factor genes CSF2 (GM-CSF) and CSF3 (Granulocyte-colony stimulating factor, G-CSF), chemokine CXCL1 and CXCL3, anti-inflammatory factor IL10, and signal transduction genes PTGS2 and SERPINE1, all had an additional ≥ 3-fold up-regulation in the CHD-FA treated wound compared to the untreated control. In addition to the well-known anti-inflammatory nature of IL10 (1, 2) and the confirmed influence of CSF2 and CSF3 on migration and proliferation of endothelial cells (3), CXCL1 and CXCL3 have also been reported to induce endothelial cell proliferation in vitro and angiogenesis in vivo (7). Therefore, the superimposed effect of a greater overexpression of these genes in the CHD-FA treated wound relative to the untreated control is consistent with an accelerated wound healing process induced by CHD-FA. Furthermore, expression profiling at day 6 revealed a reversed contrast between CHD-FA treated and untreated wounds (**Figure 6, 8**). At day 6, the expression level changes of most genes in CHD-FA treated wound were drastically reduced and approaching baseline levels, while these genes in the sham control were still gradually increasing, with the exception of IL10 of which the expression level dropped relative to day 3. The rapid restoration of differentially regulated expression level of wound healing genes in the CHD-FA treated wounds demonstrated a faster cellular response and tissue repair exerted by CHD-FA. Of note, the expression level of IL6 was gradually increased during the first 6 days of experiment and was greatly increased by >2485-fold at day 6 in the untreated wound. In contrast, in the CHD-FA treated wound, IL6 was overexpressed by 791-fold at day 3 followed by a rapid restoration of the expression from day 3 to day 6. Prolonged overexpression of IL6 is associated with impaired wound healing (4), our observation clearly demonstrated the accelerated wound healing efficacy of CHD-FA on the infected wound.

At day 6, the last rat left in the sham control group became systemically infected, thereafter; no rat was available for wound healing evaluation for day 10. We observed the same trend for gene expression from the wound treated with CHD-FA **(Figure 7**), similar to that observed from MRSA infection model.

Figure 5 Day 3 post infection wound healing gene expression profiling Untreated vs. 4.6% CHD-FA treatment in Pseudomonas infected wounds

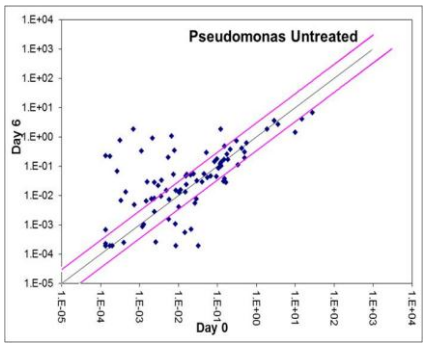


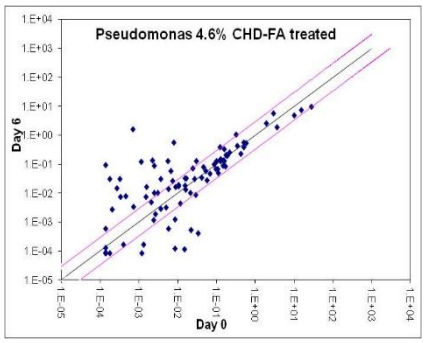


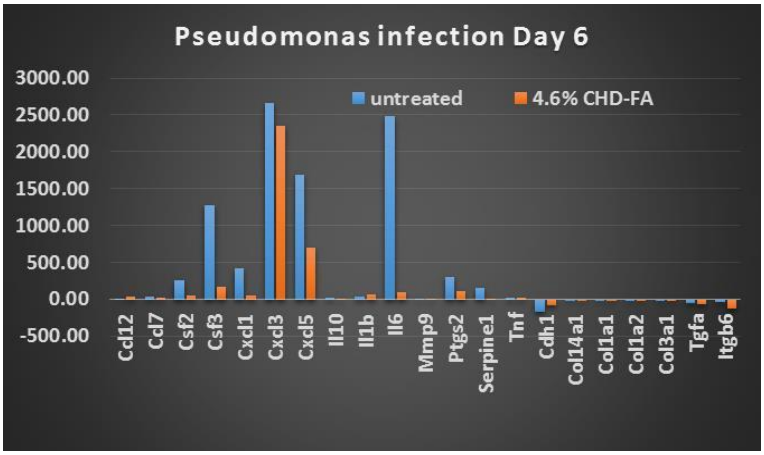


Pseudomonas Infection Day 3 vs. Day 0					
Increased expression			Decreased expression		
Fold change			Fold change		
Gene name	Untreated	4.6% CHD-FA	Gene name	Untreated	4.6% CHD-FA
Ccl12	71.95	49.15	Cdh1	-199.60	-382.95
Ccl7	44.76	31.43	Col14a1	-37.69	-197.54
Csf2	200.02	1462.28	Col1a1	-49.56	-115.44
Csf3	1030.41	2874.31	Col1a2	-28.56	-64.27
Cxcl1	187.27	836.95	Col3a1	-12.69	-31.04
Cxcl3	3080.61	8834.91	Tgfa	-51.84	-99.46
Cxcl5	571.65	544.58	Itgb6	-5.28	-12.65
Il10	56.85	228.97			
Il1b	60.72	45.70			
Il6	533.37	791.80			
Mmp9	13.12	29.32			
Ptgs2	34.51	148.98			
Serpine1	139.01	390.45			
Tnf	82.08	102.47			

Figure 6. Day 6 post infection wound healing gene expression profiling Untreated vs. 4.6% CHD-FA treatment in Pseudomonas infected wounds







Pseudomonas Infection Day 6 vs. Day 0					
Increased expression			Decreased expression		
Fold change			Fold change		
Gene name	Untreated	4.6% CHD-FA	Gene name	Untreated	4.6% CHD-FA
Ccl12	11.95	37.09	Cdh1	-170.19	-77.01
Ccl7	37.12	23.72	Col14a1	-4.78	-1.67
Csf2	254.94	56.41	Col1a1	-6.92	-2.05
Csf3	1277.40	176.44	Col1a2	-4.04	-2.79
Cxcl1	430.24	61.52	Col3a1	-3.62	-2.02
Cxcl3	2663.30	2357.43	Tgfa	-44.20	-69.17
Cxcl5	1685.55	700.87	Itgb6	-26.56	-124.24
Il10	30.25	18.29			
Il1b	45.70	71.41			
Il6	2484.95	99.25			
Mmp9	9.15	8.74			
Ptgs2	300.04	108.61			
Serpine1	165.31	8.90			
Tnf	20.24	22.05			

Figure 7. Scatter plot of wound healing gene expression of CHD-FA treated in Pseudomonas infected wounds on Day 10

No untreated group data available. All rats in the untreated group died before day 10.

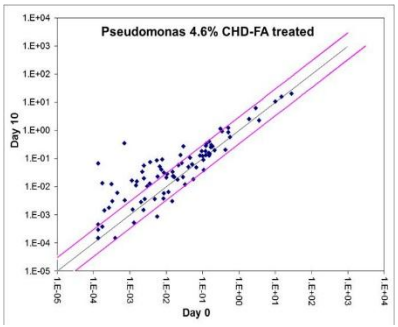
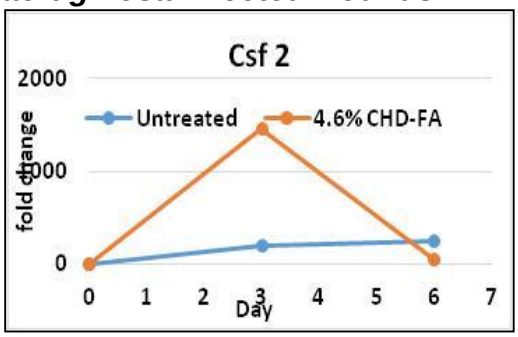
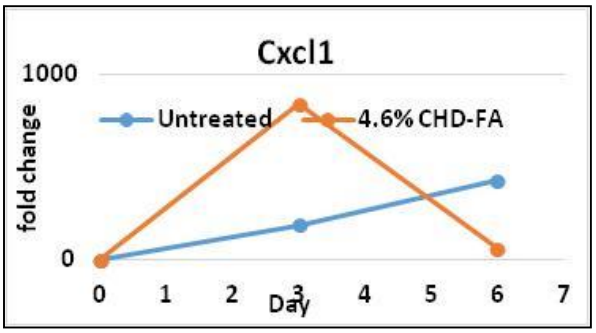
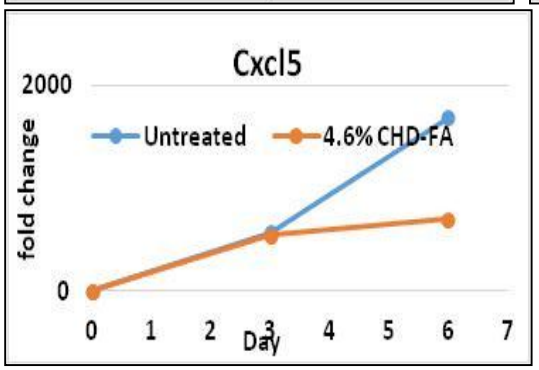
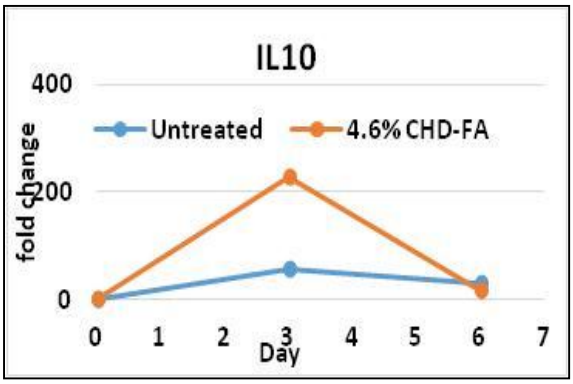


Figure 8. Temporal changes of individual wound healing gene targets for *Pseudomonas aeruginosa* infected wounds

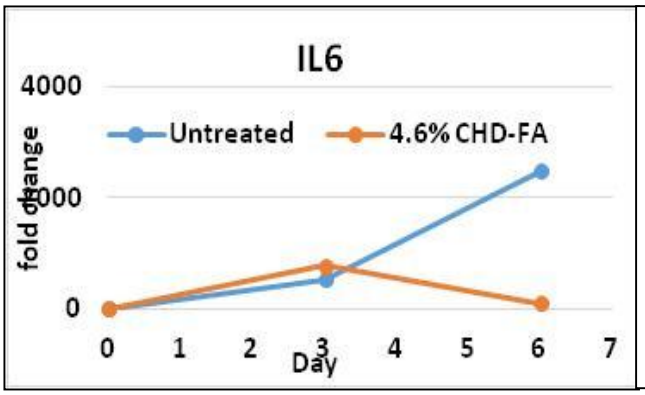








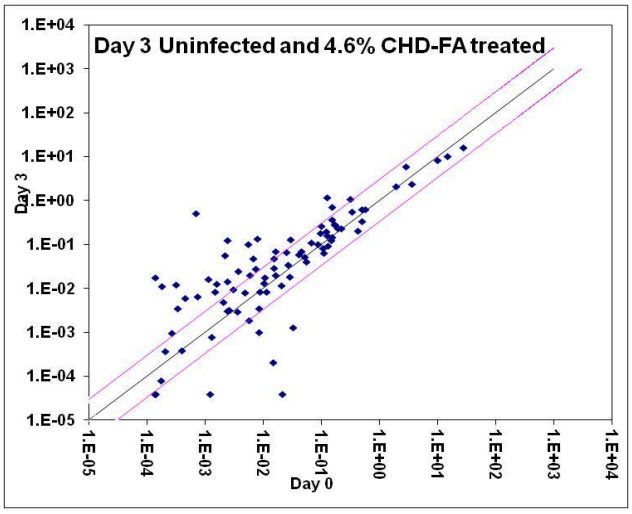
High level expression of CSF2 (granulocyte-macrophage-colony stimulating factor, GM-CSF), CXCL1, CXCL5, and anti-inflammatory IL10 influence migration and proliferation of endothelial cells leading to faster wound healing in CHD-FA treated wounds

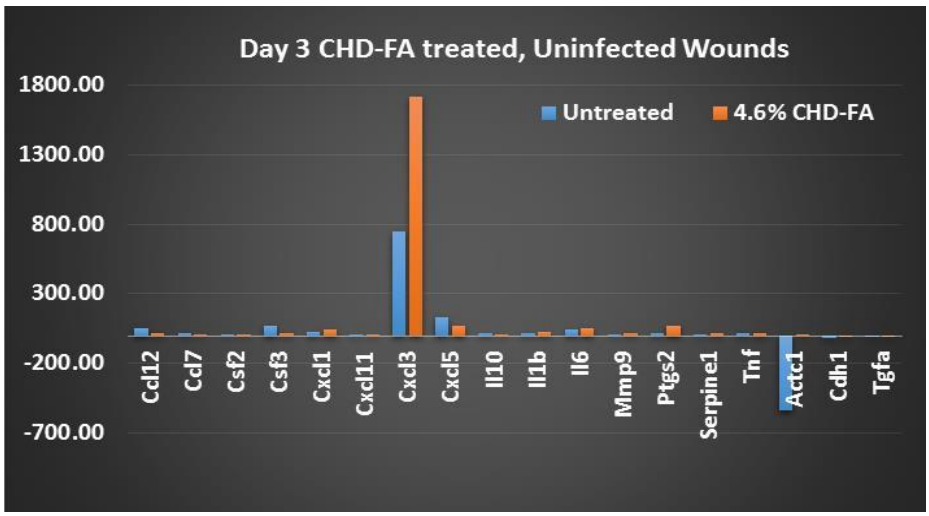


The key biomarker of impaired wound healing IL6 was constantly overexpressed through day 3 to day 6 in the untreated wound, the significantly reduced (25-fold) expression level in the CHD-FA treated wound manifested accelerated and better controlled wound healing.

CHD-FA treated uninfected wounds: Expression profiling was also assayed on uninfected wounds with or without CHD-FA treatment up to the day 3 time point. This experiment was performed to evaluate baseline expression profiling changes on the wounds due to CHD-FA. Interestingly, genes that showed significant differential expression in the uninfected wounds were very similar with those observed from the infected wounds but displayed much less fold changes relative to normal expression (**Figure 9**). Compared to untreated and uninfected wound, genes CSF3 and IL10 were overexpressed by an additional >3-fold in the CHD-FA treated and uninfected wound. Meanwhile, ACTC1, CDH1, and TGFA were significantly down-regulated to a more profound level in the CHD-FA treated relative to the untreated wound. In addition to earlier mentioned wound healing promoting markers CSF3 and IL10, down-regulation of cytoskeleton gene ACTC1 and cell adhesion gene CDH1 were also related to favorable pro-regenerative outcome by reducing contraction and promoting cell migration in the wound site. Therefore, the expression profiling on the uninfected wounds demonstrated the anti-inflammatory properties of CHD-FA. In the meantime, the comparison between uninfected, CHD-FA treated wounds and MRSA or *P. aeruginosa* infected, CHD-FA treated wounds showed that the infected wounds had much more prominent (> 10-fold) gene expression changes, suggesting that the expression changes observed from the infected rat model were actually indicative to a much larger extent of an anti-infection progress relative to that of the anti-inflammatory properties of CHD-FA on uninfected wounds.

Figure 9. Day 3 wound healing gene expression for 4.6% CHD-FA treated uninfected wounds





No Infection Day 3 vs. Day 0					
Increased expression			Decreased expression		
Fold change			Fold change		
Gene name	Untreated	4.6% CHD-FA	Gene name	Untreated	4.6% CHD-FA
Ccl12	53.11	10.32	Actc1	-537.08	6.67
Ccl7	18.58	8.53	Cdh1	-24.74	-1.33
Csf2	3.62	2.91	Tgfa	-8.31	-1.26
Csf3	64.04	10.91			
Cxcl1	26.56	44.85			
Cxcl11	8.91	2.09			
Cxcl3	750.13	1718.58			
Cxcl5	129.88	68.93			
Il10	13.56	4.43			
Il1b	17.58	20.85			
Il6	39.97	46.11			
Mmp9	6.76	16.02			
Ptgs2	14.73	67.04			
Serpine1	7.37	15.32			
Tnf	10.71	15.11			

Conclusion

Wound healing genes displayed a similar pattern of differential expression over the course of wound healing for both MRSA and *P. aeruginosa* infected rats. In the absence of infection, the differentially expressed genes were the same as that observed in the infected model but at much lower level. In addition to the antimicrobial behavior of CHD-FA, this data is consistent with previous reports that CHD-FA has prominent anti-inflammatory properties. It is noticeable that CHD-FA treated wounds were primed to progress to wound healing in both infection models. For instance, IL10, an important anti-inflammatory cytokine (1, 2), was more highly expressed in the early infection relative to the untreated control. Thus, CHD-FA treated wounds were more likely to progress to the next stage of healing due to reduced inflammation. At the endpoint, most of the genes were restored to baseline expression levels in the CHD-FA treated wounds. However, in contrast, pro-inflammatory cytokine IL6 and matrix metalloproteinase MMP9 were still highly expressed in the untreated wounds. CHD-FA prominently blocked the overexpression of IL6 at the mid-point of infection. Given that both prolonged over expression of IL6 and MMP9 are associated with impaired wound healing (4-7), such data are consistent with histopathological data and provide further evidence of enhanced wound healing effect of CHD-FA.

Key Research Accomplishments

- **A complete profile of antibacterial and antifungal properties of CHD-FA as defined in specific aim 1 of the statement of work.**
- **Established a reproducible cutaneous wound model in rats for bacteria and fungi that can be used to assess CHD-FA to treat wound infections**
- **Obtained comprehensive data on CHD-FA to treat cutaneous MRSA and *P. aeruginosa* wound infections in rats.**
- **Obtained preliminary efficacy data on CHD-FA to treat cutaneous *Acinetobacter baumannii, E. coli and K. pneumonia and Aspergillus fumigatus* wound infections in rats.**
- **Obtained histopathological analysis of wounds from rats infected with MRSA and treated with CHD-FA.**
- **Demonstrated CHD-FA blocks cellular inflammation and increases epithelialization, consistent with improved wound healing.**
- **Performed host gene expression profiling of MRSA and *P. aeruginosa* infected cutaneous wounds, and demonstrated CHD-FA blocks cellular inflammation and consistent with improved wound healing.**

Reportable Outcomes

The CHD-FA efficacy results entitled **"CHD-FA is a highly effective topical broad-spectrum antimicrobial for drug-resistant wound infections"** was presented as a poster at the 54th Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC 2014, Washington DC). Dr. Perlin has also attended the Military Health System Research Symposium (MHSRS) 2014 in August and gave an oral presentation entitled "**Evaluation of Carbohydrate Derived Fulvic Acid (CHD-FA) as a Topical Broad-Spectrum Antimicrobial for Drug Resistant Wound Infections"**. A cutaneous wound model in rats for MRSA and *P. aeruginosa*, *Acinetobacter baumannii, E. coli and K. pneumonia* and pathogenic mold *Aspergillus fumigatus* was developed and validated. Histopathological analysis of wounds from rats infected with MRSA and *P. aeruginosa* and treated with CHD-FA at 4h post infection was performed during this reporting cycle. Host gene expression profiling assessing wound healing genes was also performed for MRSA and *P. aeruginosa* infected wounds in rats.
Dr. Perlin was awarded a NIH Research Program – Cooperative Agreements (U19) grant (RFA-AI-12-044) that resulted in PHRI-Rutgers University being designated as one of the NIAID Centers for Excellence in Translational Research (CETR) to develop novel leads against drug resistant pathogens. The wound model was a significant component in the assessment of novel lead compounds against drug resistant bacterial pathogens.

Conclusion

In this second annual report, a cutaneous wound model in rats was established and infection models with *Escherichia coli*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, MRSA, *Pseudomonas aeruginosa* and *Aspergillus fumigatus* were validated. Our initial results demonstrate that CHD-FA can reduce microbial burdens of susceptible and drug-resistant planktonic bacteria and fungi, and significantly improved wound healing kinetics. Histopathological and host gene expression profiling analyses of wounds infected with *P. aeruginosa* and MRSA and treated with CHD-FA demonstrated improved healing at the cellular and molecular level. Specifically, CHD-FA treated wounds displayed decreased cellular inflammation, improved angiogenesis and fibroblast scores and increased rates of epithelialization. Host expression profiling of wound healing genes showed similar behavior with CHD-FA treated wounds with a prominent effect of reducing inflammation. During the next quarter and throughout the year, we will perform both histopathological and host gene expression profiling to assess the cellular and molecular mechanisms during wound healing with CHD-FA against the other pathogens listed in the statement of work. We will also perform the other wound infection models such as the burn model and deep tissue (thigh) model. Our studies have raised some open questions. For example, the inconsistencies observed in the wound burden reduction. This may be due to the presence of mixed microbial biofilm within the scab that prevents a significant reduction in burden in some models. Overall, we have made strong progress in the second year. We are optimistic that CHD-FA will be effective in other models and demonstrate its universality for preventing wound infections and promoting healing.

References

1. Belperio JA, Keane MP, Arenberg DA, Addison CL, Ehlert JE, Burdick MD, Strieter RM. CXC chemokines in angiogenesis. Journal of leukocyte biology. 2000;68(1):1-8.
2. Orman MA, Nguyen TT, Ierapetritou MG, Berthiaume F, Androulakis IP. Comparison of the cytokine and chemokine dynamics of the early inflammatory response in models of burn injury and infection. Cytokine. 2011;55(3):362-71.
3. Bussolino F, Wang JM, Defilippi P, Turrini F, Sanavio F, Edgell CJ, Aglietta M, Arese P, Mantovani A. Granulocyte- and granulocyte-macrophage-colony stimulating factors induce human endothelial cells to migrate and proliferate. Nature. 1989;337(6206):471-3.
4. Shah JM, Omar E, Pai DR, Sood S. Cellular events and biomarkers of wound healing. Indian journal of plastic surgery : official publication of the Association of Plastic Surgeons of India. 2012;45(2):220-8.
5. Chen L, Arbieva ZH, Guo S, Marucha PT, Mustoe TA, DiPietro LA. Positional differences in the wound transcriptome of skin and oral mucosa. BMC genomics. 2010;11:471.
6. Ladwig GP, Robson MC, Liu R, Kuhn MA, Muir DF, Schultz GS. Ratios of activated matrix metalloproteinase-9 to tissue inhibitor of matrix metalloproteinase-1 in wound fluids are inversely correlated with healing of pressure ulcers. Wound repair and regeneration : official publication of the Wound Healing Society [and] the European Tissue Repair Society. 2002;10(1):26-37.
7. Norgauer J, Hildenbrand T, Idzko M, Panther E, Bandemir E, Hartmann M, Vanscheidt W, Herouy Y. Elevated expression of extracellular matrix metalloproteinase inducer (CD147) and membrane-type matrix metalloproteinases in venous leg ulcers. The British journal of dermatology. 2002; 147(6):1180-6.

Appendices

Updated Quad Chart

Evaluation of Carbohydrate Derived Fulvic Acid (CHD-FA) as a Topical Broad-Spectrum Antimicrobial for Drug Resistant Wound Infections

Insert ERMS/Log Number and Task Title Here

W81XWH-11-DMRDP-MID-ARA

PI: David S. Perlin Org: New Jersey Medical School-Rutgers, The State University of New Jersey Award Amount: $1,530,400

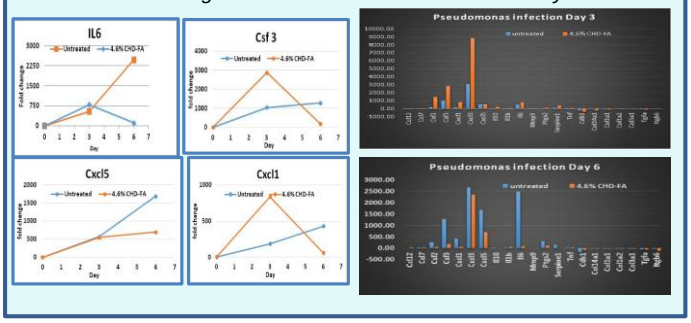
Study/Product Aim(s)

- **Assess *in vitro* susceptibility of CHD-FA against multi-drug resistant bacteria and fungal pathogens**
- **Evaluate efficacy of topical application of CDH-FA on small animal models of wound infection with a wide variety of bacterial and fungal pathogens.**

Approach

Establish minimum inhibitory concentrations (MIC_{50} and MIC_{90}) for CDH-FA against large collections of clinical isolates representing wound-associated drug resistant bacteria and fungi. Rat models of wound infection (open, burn and deep tissue) will be established with healthy animals using drug resistant bacterial and fungal pathogens. Increasing doses of CDH-FA in topical form will be used to assess relative efficacy.

Host gene expression profiling to assess wound healing of CHD-FA treated P. aeruginosa infected rat skin wounds on day 3 and 6



The key biomarker of impaired wound healing IL6 was constantly over expressed through day 3 to day 6 in the untreated wound. The significantly reduced (25-fold) expression level of IL6 in the CHD-FA treated wound manifested in accelerated and improved wound healing.

Timeline and Cost

Activities / CY	12	13	14	15
Establish MIC_{90}s for CHD-FA with clinical isolates of major drug resistant pathogens				
Assess CHD-FA in animal models of wound infection for major pathogens				
in vivo evaluation of CDH-FA in open, burn and deep tissue models.				
Histopathological analysis and gene expression profiling.				
Estimated Budget ($K)	**$400**	**$600**	**$500**	**$400**

Updated: October 29, 2014

Goals/Milestones

CY12 Goal – Establish (MIC_{50} and MIC_{90}) for CDH-FA

☑ *in vitro* efficacy of CHD-FA against drug resistant bacteria.

CY13 Goals – Complete *in vitro* susceptibility testing and establish cutaneous wound model in rats.

☑demonstrated the potent anti-microbial activity of CHD-FA against a broad range of drug resistant bacteria and fungi.

CY14 Goal – Establish open/ burn wound infection model in rats using drug resistant bacterial and fungal pathogens following treatment with CDH-FA in topical form.

☑ established the cutaneous wound model in rats with major pathogens.

CY15 Goal – *in vivo* evaluation of CHD-FA in deep tissue models in rats. Perform both histopathological analyses and host gene expression profiling to assess wound healing.

Comments/Challenges/Issues/Concerns

- Inconsistent bacterial burden reduction observed in infected wounds between various treatment groups.

Budget Expenditure to Date

Actual Expenditure:$ 917,239

Quarterly technical report Q5.01.2014

"Evaluation of Carbohydrate-Derived Fulvic Acid (CHD-FA) as a Topical Broad-Spectrum Antimicrobial for Drug-Resistant Wound Infections."

Quarterly Technical Progress Report (Q5.01.2014)

Performance period: 09/30/2013 through 12/30/2013.

Date of report: January 9, 2014

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Quarterly technical report Q6.04.2014

"Evaluation of Carbohydrate-Derived Fulvic Acid (CHD-FA) as a Topical Broad-Spectrum Antimicrobial for Drug-Resistant Wound Infections."

Quarterly Technical Progress Report (Q6.04.2014)

Performance period: 12/31/2013 through 03/30/2014.

Date of report: April 16, 2014

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Quarterly technical report Q7.07.2014

"Evaluation of Carbohydrate-Derived Fulvic Acid (CHD-FA) as a Topical Broad-Spectrum Antimicrobial for Drug-Resistant Wound Infections."

Quarterly Technical Progress Report (Q7.15.2014)

Performance period: 4/1/2014 through 06/30/2014.

Date of report: July 15, 2014

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